Sleep-Dependent Declarative Memory Consolidation and Its Application to Emotional and Extinction Memory Processing

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Abstract

The active role of slow-wave sleep (SWS) in declarative memory consolidation has been evidenced in many studies and spontaneous hippocampal reactivations are assumed as underlying neural mechanism. This mechanism is usually investigated with targeted memory reactivation (TMR) which is applied by associating a background stimulus with a learning task and re-presenting this cue during post-learning sleep. The five studies presented in this dissertation further investigate the declarative memory-enhancing effect of SWS and apply it to other memory types.

In the first study, we replicated the beneficial effect of olfactory TMR on memory consolidation and could show that changes in sleep EEG parameters associated with memory indicated a bottom-up influence of post-learning hippocampal memory replay on cortical slow oscillations and thalamo-cortical sleep spindles.

We found piriform cortex hyporesponsiveness to olfactory stimuli in sleep compared with wakefulness in the second study, which opened the possibility of a stronger involvement of other functionally connected regions in olfactory processing in sleep. However, no other brain regions, such as amygdala or hippocampus, were higher activated during odor presentation in sleep compared with wakefulness.

In the third study, we did not observe a beneficial effect of TMR during late REM sleep on emotional memory processing, questioning the role of covert emotional memory reactivations during sleep as possible underlying mechanism.

We found an increase in memory-related EEG parameters after TMR of extinction memory in anxiety patients in the fourth study. However, there were no effects of TMR on post-sleep fear behavior, probably due to a ceiling effect in extinction learning.

The fifth study revealed an increase in the connectivity between dorsal nexus and dorsolateral prefrontal cortex after sleep deprivation compared with normal wakefulness in a healthy sample. These findings need to be replicated in a clinical population.

In sum, these studies confirm the declarative memory-enhancing effect of SWS and provide information about its underlying neural mechanisms. Furthermore, they apply it to emotional processing, emotional memory processing, and extinction memory processing. We could not confirm a critical role in covert memory reactivations during REM sleep as an underlying mechanism of sleep-dependent emotional memory processing. In contrast to these findings, we could partly confirm an effect of TMR on extinction memory. This involves a

great potential for the application to clinical praxis and needs to be further replicated and specified by future studies.

Zusammenfassung

Mehrere Studien unterstreichen die aktive Rolle des Tiefschlafs (slow-wave sleep, SWS) in der Konsolidierung des deklarativen Gedächtnis‘. Spontane hippocampale Reaktivierungen werden als neuronaler Mechanismus dahinter vermutet. Dieser Mechanismus wird üblicherweise mit exogener Gedächtnisreaktivierung untersucht (targeted memory reactivation, TMR), wobei ein Hintergrundreiz mit einer Gedächtnisaufgabe assoziiert und im SWS erneut dargeboten wird. In den fünf in dieser Dissertation präsentierten Studien wird der förderliche Effekt von SWS auf deklaratives Gedächtnis untersucht und auf andere Gedächtnistypen angewandt.

In der ersten Studie replizierten wir den förderlichen Effekt olfaktorischer TMR auf Gedächtniskonsolidierung. Veränderungen in gedächtnisassoziierten Schlaf-EEG Charakteristiken lassen einen aufsteigenden Einfluss der hippocampalen Gedächtnisreaktivierungen auf kortikale langsame Wellen und thalamo-kortikale Spindeln vermuten.

Es zeigte sich eine reduzierte Aktivierung des piriformen Kortex‘ im Schlaf verglichen mit dem Wachzustand in der zweiten Studie, was darauf hindeutet, dass andere, mit dem Geruchskortex funktionell assoziierte Hirnregionen eine stärkere Rolle bei der Geruchsverarbeitung im Schlaf einnehmen. Allerdings waren andere Hirnregionen, wie Hippocampus oder Amygdala, während der Geruchsdarbietung im Schlaf nicht höher aktiviert.

In der dritten Studie fanden wir keinen förderlichen Effekt von TMR im rapid eye movement (REM) Schlaf auf emotionale Gedächtnisverarbeitung, was die Annahme von Mechanismen verdeckter Reaktivierung emotionaler Gedächtnisinhalte im Schlaf in Frage stellt.

In der vierten Studie beobachteten wir einen Anstieg der in Gedächtniskonsolidierung involvierten EEG Charakteristika nach TMR des Extinktionsgedächtnis‘ bei Angstpatienten, der sich jedoch nicht im Verhalten widerspiegelte und wahrscheinlich durch einen Deckeneffekt bedingt ist.

Die fünfte Studie zeigte eine erhöhte Konnektivität zwischen dorsalem Nexus und präfrontalem Kortex in einer gesunden Stichprobe nach Schlafdeprivation im Vergleich zu normalen Wachsein. Diese Ergebnisse geben Einsicht in die neuronalen Mechanismen der emotionalen Verarbeitung, bedürfen aber einer Replikation an einer klinischen Stichprobe.

Diese Studien bestätigen den förderlichen Effekt von SWS auf das deklarative Gedächtnis und geben Aufschluss über zu Grunde liegende neuronale Mechanismen. Dieser Effekt wird zusätzlich auf Emotionsverarbeitung, emotionale Gedächtnisverarbeitung und Extinktionsgedächtnisverarbeitung ausgeweitet. Wir konnten keine kritische Rolle von verdeckten Gedächtnisreaktivierungen während des REM Schlafs bei schlafabhängiger emotionaler Gedächtniskonsolidierung bestätigen. Jedoch konnten wir teilweise einen Effekt von TMR auf das Extinktionsgedächtnis bestätigen. Dies birgt ein großes Potential für die Anwendung in der klinischen Praxis, bedarf allerdings weiterer Replikation und Spezifikation.

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1 Introduction

“Memory is the glue that holds our mental life together. Without its unifying power, both our conscious and unconscious life would be broken into as many fragments as there are seconds in the day.” (Kandel, Dudai, & Mayford, 2014)

This citation stretches out the importance of memory. Every experience in our life that forms our identity can only be integrated in our concept of our self if we remember it. Therefore, memory was always a topic that fascinated researchers, and systematic research about its mechanisms was already conducted almost 130 years ago, by Hermann Ebbinghaus (1885). He compared forgetting after different time intervals, and his experiments already showed that memory decay was diminished after including sleep in this time interval. Even though Ebbinghaus did not extend research on this findings, this was the impetus to systematically investigate the effect of sleep on memory, and a first study found superior memory retrieval after sleep (Jenkins & Dallenbach, 1924). The research on the relation between sleep and memory continued and is still a hot topic in science today, almost 100 years later. In contrast to the early beginning of this research field, when the beneficial effect of sleep on memory was explained by a passive protection against interference on newly learned memory traces, nowadays, the assumption that sleep plays an active role in memory processing is acknowledged, as shown by a vast amount of reviews on this subject (e.g. Abel, Havekes, Saletin, & Walker, 2013; Antony, Gobel, O’Hare, Reber, & Paller, 2012; Born, Rasch, & Gais, 2006; Diekelmann & Born, 2010; Diekelmann, 2014; Rasch & Born, 2013).

Different sleep oscillations have been implicated in the active role of sleep on memory. Yet, these mechanisms need refinement and replication, especially in human studies, since a lot of our knowledge about the neural correlates of this effect is generated by cell recordings in animals.

Furthermore, the sleep-dependent effect on memory consolidation was investigated in studies using neutral, declarative memory. Not surprisingly, an effect of sleep was also found for the processing of other memory types, such as emotional memory (e.g. Baran, Pace-Schott, Ericson, & Spencer, 2012; Hu, Stylos-Allan, & Walker, 2006; Menz et al., 2013; Wagner, Gais, & Born, 2001) or extinction memory (Hauner, Howard, Zelano, & Gottfried, 2013; Kleim et al., 2013; Pace-Schott, Verga, Bennett, & Spencer, 2012). However, findings concerning these memory types are ambiguous and do often not investigate the underlying neural correlates.

In the following sections, memory, sleep, and their interaction will be presented in more detail and questions emerging from the gaps in literature will be inferred.

1.1 Memory

The concept of our own identity critically depends on keeping important autobiographical everyday-life events in mind and remembering events in the past, as already stated in the citation of Kandel and colleagues (2014) above. Patient Henry Molaison, also known as "H. M." in his lifetime, has become a prominent example for the inability to remember large parts of his own biography. Because of serious, reoccurring epileptic seizures, surgeons decided to bilaterally remove the source of the seizures: a brain structure termed hippocampus, after the resemblance of its shape with that of a seahorse. After the surgery, the seizures disappeared, and with them Henry Molaison's ability to remember events that had happened before the intervention. Furthermore, he was not able to keep new information in mind. Other patients with hippocampal removal showed similar amnesia, with the severity of symptoms correlating with the extend of tissue removal (Scoville & Milner, 1957). Since then, the involvement of the hippocampus in memory formation is acknowledged. Intense testing of these patients revealed that they were neither able to remember events and facts in a certain time frame before the surgery, nor were they able to acquire novel facts and events after the surgery. However, the acquisition and retrieval of other aspects of memory were not affected, for example tracing a shape with their hand only visible in a mirror. This illustrates that there are different memory systems operating largely independent of each other and that they are, importantly, associated with different brain structures. To shed further light on these processes, the different memory systems and their representation in the brain will first be described in the following. Thereafter, the process of memory formation will be explained.

1.1.1 Memory systems

There are multiple classifications of the broad term "memory", since memory consists of many sub-types. A common way to classify memory depends on the time the information is stored in the brain: for a short time, or for a long time. The division according to time is classified in sensory buffers, short-term memory, and long-term memory (Atkinson & Shiffrin, 1968). Sensory buffers for each sensory system hold information for several seconds and can be considered as filters that prevent the brain from sensory overload. Short-term

memory stores information for several seconds or – with means of rehearsal – minutes or even hours. The storage system of information for hours, days, years, and even a life-time is considered as long-term memory (Atkinson & Shiffrin, 1968). Further, long-term memory is subdivided in so called declarative, or explicit, and non-declarative, or implicit, memory (Squire & Zola, 1996; Squire, 1992; see Figure 1).

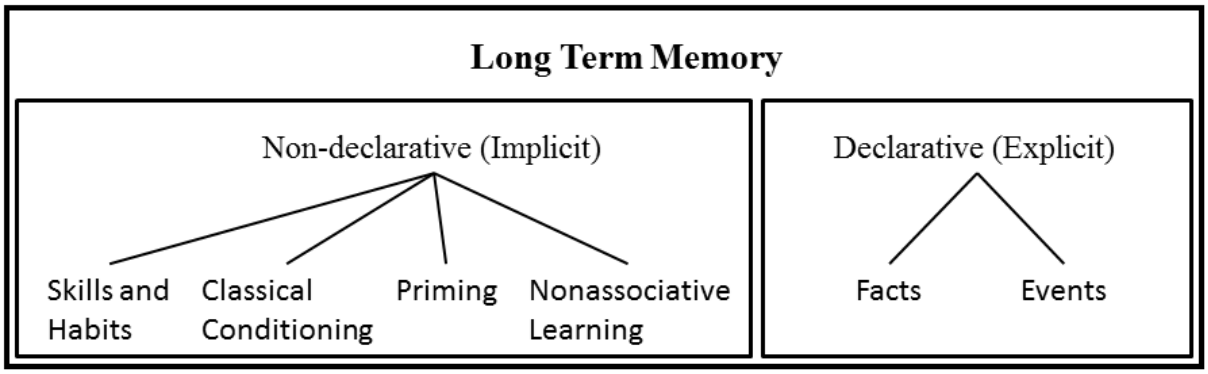

Figure 1. Classification of long term memory into different memory systems.
Long term memory can be classified into non-declarative and declarative memory. The subtypes of non-declarative memory are skills and habits, classical conditioning, priming, and nonassociative learning, whereas declarative memory is subdivided in memory for facts and memory for events. Adapted from Squire & Zola (1996).

Non-declarative memory does not require conscious awareness to be formed or retrieved and can further be subdivided in four different memory systems (for a recent review see Henke, 2010): skills and habits, classical conditioning, priming, and non-associative learning. Memory for skills and habits, also termed "procedural memory", comprises motoric memory. It is usually acquired by repetition and practice, for example swimming. Not surprisingly, this memory depends on the basal ganglia, a brain structure involved in motoric processes (Henke, 2010). Classical conditioning contains information about an association. A well-known example for this memory was investigated in dogs: the repeated pairing of the ring of a bell with food. After several presentations, the association between the two stimuli was successfully established, and the ring of the bell alone caused salivation, the reaction usually observed with food (Pavlov, 1927). This subform of non-declarative memory is also called "Pavlovian conditioning", after its discoverer. The brain structures involved in this memory type are the cerebellum and the amygdala (Henke, 2010). Priming is the facilitation of processing a stimulus when its features are familiar due to prior exposure to the same or a similar stimulus, for example recognizing a word based on the presentation of some letters. It

depends on the neocortex (Henke, 2010). Non-associative learning compasses habituation and sensitization, memory in response to repeated stimuli presentation, for example a non-conscious adjustment to loud sounds. This memory type depends on reflex pathways (Castellucci, Pinsker, Kupfermann, & Kandel, 1970; Henke, 2010).

In contrast to implicit non-declarative memory, declarative memory requires conscious awareness of the memory content and comprises the knowledge about facts, also called semantic memory, and autobiographical events anchored in space and time, also called episodic memory.

An example for semantic memory is the knowledge about the fact that Chicago is a city in the United States of America, whereas an example for episodic memory is the context of one's first kiss. On the neural level, this declarative information is assumed to be encoded and initially stored in the hippocampus and the medial temporal lobe (Eichenbaum, 2000; Squire & Zola, 1996; Squire, 1992).

However, for episodic declarative memories, there seems to be a difference in the formation as well as in the neural mechanisms depending on its affective components. Events that are accompanied by high arousal and valence, the two dimensions of emotion (LaBar & Cabeza, 2006), usually are remembered better than neutral ones (Cahill & McGaugh, 1998; McGaugh, 2004, 2006). On a neurobiological level, this effect during emotional compared with neutral experience can be attributed first to the release of different neuromodulators usually associated with stress, such as noradrenalin and glucocorticoids, and second to the additional activation of brain structures during encoding, amongst others the amygdala (McGaugh, 2004). This special form of episodic declarative memory needed to be classified and outlined here because it will form an important part of the thesis. However, when memory formation will be explained more detailed in the following, the term declarative memory will conventionally refer to neutral declarative memory.

To come back to neutral declarative memory, the important role of the hippocampus was first evidenced by neurological patients with hippocampal removal (Scoville & Milner, 1957). Importantly, these patients could retrieve declarative memories which had been formed long time before their surgery. This indicates that memory is not based on the hippocampus alone, but seems to undergo a dynamic process of reorganization during which it becomes more and more hippocampus-independent over time. This interesting finding of plasticity in the memory system is part of memory formation and will be further explained in the following section.

1.1.2 Memory formation

Memory formation consists of three stages of information processing: learning, consolidation, and retrieval. The new memory is encoded during learning, stabilized during consolidation, and consciously or unconsciously recalled during retrieval. Whereas the first and the last stage of memory formation are most effective during wakefulness, consolidation seems to profit rather from sleep (Rasch & Born, 2013). Therefore, the next paragraphs will only shortly outline learning and retrieval and focus more detailed on the mechanisms of consolidation.

As already mentioned above, learning can take place in an explicit or implicit way. An example for predominant implicit learning is the acquisition of the native language during childhood, whereas learning vocabularies of a foreign language is an example for explicit learning. The brain state during which learning usually occurs is wakefulness, although recent studies surprisingly found that it is possible to learn novel tone-odor or odor-odor associations during sleep, without even knowing about the learning procedure (Arzi et al., 2012, 2014). Despite these findings, it is assumed that learning is most effective during wakefulness, since it seems questionable that other forms of memory besides implicit association learning are possible during sleep.

There are also different kinds of retrieval: retrieval without the learned stimulus, termed free recall, and retrieval after the presentation of a stimulus that was associated with the learning procedure, termed cued recall. The third way of retrieval is correctly identifying a previously learned object, termed recognition. Free recall takes place in everyday life, for example in a supermarket when we have forgotten our grocery list and need to reconstruct the list in mind. However, in this situation, cued recall can also occur when we see items on the shelves that we have written on the list. Another example for cued recall is the emerging memory of previous holidays while looking at pictures taken during this time. In an experimental setting, these different types of retrieval could be constructed by asking participants to write down every item they remember of a previously learned list or a set of pictures for free recall, presenting one word of a previously learned word pair association for cued recall, and presenting a picture or a word and asking if this stimulus was in the previously learned word list or picture set. However, memory does not only depend on learning and retrieval, but also on the stage in-between: consolidation.

The term consolidation traces back to 1900, when Müller and Pilzecker detected that encoding a stimulus does not necessarily lead to a successful retrieval of this stimulus, evidenced by a series of memory experiments in healthy participants (Müller & Pilzecker,

1900). They concluded that newly acquired memories are vulnerable to interference and need to be fixated or consolidated in order to persist and to be accessible to retrieval.

Nowadays, the term consolidation is conventionally used to describe two different processes: synaptic consolidation and system consolidation (Dudai, 2004). Consolidation on the synaptic level takes place minutes to hours after encoding, whereas system consolidation requires a longer time frame.

On a synaptic level, the neurobiological basis of the stabilization of memory is "long-term potentiation" (LTP). The theoretical assumption that synapses between two cells form a stronger connection if they are active at the same time (Hebb, 1949) stimulated the discovery of this process. Evidence for this mechanism in the mammalian brain came from the observation of a strengthening of hippocampal synapses when two cells fired together. This was investigated by applying bursts of high-frequency electric shocks to the synaptic pathway (Bliss & Collingridge, 1993; Bliss & Gardner-Medwin, 1973; Bliss & Lomo, 1973). However, this well-acknowledged concept of synaptic consolidation describes memory formation only in the short-term range. It cannot account for retrograde amnesia for events shortly before the surgery in neuropsychological patients after hippocampal removal, such as observed in H. M. It seems therefore that memory is not simply stored in the hippocampus, but undergoes a dynamic process.

The "two-stage model", a model based on long-time reorganization of hippocampal information due to a transfer to the neocortex, was proposed by Marr (1971). In detail, newly acquired memories are first encoded in parallel in the hippocampus and in specialized parts of the primary and associative neocortex. Thereby, the hippocampus binds the different aspects of the information distributed in the different cortical regions (Eichenbaum, 2004). Spontaneous hippocampal reactivation reinstates simultaneous activity in the different cortical areas via hippocampal-cortical pathways. This in turn strengthens cortico-cortical connections between the activated regions. The result is an emerging hippocampal-independence of the memory trace and an integration of the memory trace into already existing networks (Frankland & Bontempi, 2005; Marr, 1971). Importantly, it is also assumed that the consolidation at a system level is most effective during sleep (Buzsáki, 1989; Marr, 1971). Therefore, sleep will be the topic of the next paragraph.

1.2 Sleep

Sleep is a behavioral state that is mostly defined as reversible, with unresponsiveness to external stimuli, perceptive disengagement, physiological inactivity, and the loss of consciousness (Carskadon & Dement, 2011; Rasch & Born, 2013). Remarkably, despite this loss of consciousness, our brain is still very active during sleep. Therefore, sleep is considered a dynamic brain state, as evidenced by different, alternating sleep stages and sleep cycles. In order to identify these different sleep stages, typically recordings from polysomnography (PSG) are used, combining the information of signals of spontaneous electrical brain activity measured by electroencephalography (EEG), of eye movements (electrooculography, EOG), and of muscle activity (electromyography, EMG). Each sleep stage consists of a unique PSG profile. To analyze the composition of a night of sleep period, epochs of sleep PSG data (conventionally with a length of 30 s) are classified as one sleep stage based on these characteristics. This process creates an individual "hypnogram", a figure that depicts the different sleep stages over the sleep period (see Figure 2). The following paragraph aims at describing the different sleep stages and outlining their characteristics.

1.2.1 Sleep stages

Typically, a normal night of sleep consists of different sleep cycles with durations of approximately 90 minutes. Within one cycle, non-rapid eye movement (NREM) and rapid eye movement (REM) sleep stages alternate. The structure of the different sleep cycles changes during the night: In early sleep, NREM sleep stages are predominant, whereas later sleep cycles mostly contain REM sleep (see Figure 2). The sleep stages constituting NREM sleep are NREM sleep stage 1 (N1), NREM sleep stage 2 (N2), and NREM sleep stage 3 (N3), the latter also termed as slow-wave sleep (SWS). REM sleep is a unique sleep stage without further subdivision (Iber, Ancoli-Israel, Chesson, & Quan, 2007). In the following paragraph, these sleep stages and their characteristics are described.

The classification of the different sleep stages based on unique PSG events of each sleep stage is defined by the manual of the American Academy of Sleep Medicine (Iber et al., 2007). All of the following rules for scoring sleep are adapted from this source. According to this manual, the wake state is defined by predominant alpha frequency in the EEG between 8 and 13 Hz and eye blinks. Sleep starts when the first PSG epoch is scored different than wake, which is N1 in most of the cases. To score a PSG epoch as N1, it must contain low amplitude electrophysiological activity between 4 and 7 Hz, and vertex waves which are distinctive,

sharp waves with less than half a second. Furthermore, slow, regular, sinusoidal eye movements with deflections of more than half a second duration must be visible in the EOG. Sleep stage N2 is hallmarked by two characteristic electrophysiological events: K complexes and spindles. K complexes are clearly visible, sharp, negative waves with durations longer than half a second. Spindles are EEG waves that are also longer than half a second in a frequency range between 11 and 16 Hz. N3 or SWS is characterized by at least 20% of the epoch consisting of very slow electrophysiological activity with oscillations between 0.5 and 2 Hz and amplitudes higher than 75 µV, low muscle activity, and the absence of eye movements. The last sleep stage, REM sleep, is characterized especially by prominent, irregular rapid eye movements, low muscle tonus, and low amplitude and mixed frequencies EEG (Iber et al., 2007).

In addition to the above mentioned polysomnographic differences between the different sleep stages, NREM sleep and REM have also distinct profiles of neuromodulator levels (Born et al., 2006; Diekelmann & Born, 2010). While acetylcholine (Hasselmo, 1999) and cortisol (Born & Fehm, 2000; Born, Hansen, Marshall, Mölle, & Fehm, 1999) concentrations are low during NREM sleep, they attain high, almost wake-like, concentrations during REM sleep. By contrast, hormones like noradrenaline are low concentrated during REM sleep, show intermediate levels during NREM sleep and reach a peak during wakefulness (Hasselmo, 1999; Lestienne, Hervé-Minvielle, Robinson, Briois, & Sara, 1997).

As already mentioned, some characteristics of sleep, namely slow oscillations, sharp wave-ripples, and sleep spindles, seem to hold an important role in memory processing. Therefore, they will be presented more detailed in the following paragraphs.

1.2.2 Sleep oscillations

There are some oscillations that typically occur during sleep: neocortical slow oscillations, hippocampal sharp wave-ripples, and thalamo-cortical spindles. All of them have been implicated in memory consolidation during sleep. Therefore, knowledge about their underlying biology is important for understanding the interaction of sleep and memory. This will be described in the following sections.

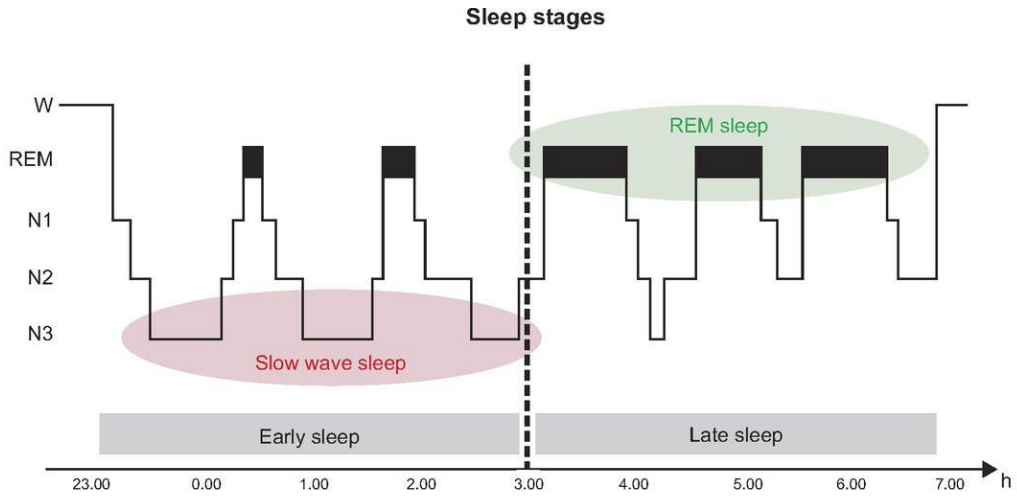

Figure 2. Sequence of sleep stages during nocturnal sleep.
The cyclic alterations of NREM sleep (with sleep stages N1, N2, and N3) and REM sleep during nocturnal sleep differ in their composition of sleep stages. The first half of the night is predominated by NREM sleep, whereas REM sleep is most dominant during the second half of the night. Adapted from Rasch & Born (2013).

Slow wave activity and slow oscillations

The predominant EEG spectral power band during SWS consists of frequencies between 0.5 to 4.0 Hz (Rasch & Born, 2013) and is also referred to as "slow wave activity" (SWA). It includes slow oscillations between 0.5 and 1.0 Hz with a peak at 0.7 to 0.8 Hz (Achermann & Borbély, 1997). They reflect rhythmic, synchronous electrophysiological states of alternating activity and silence (Contreras & Steriade, 1995, 1996; Steriade, Timofeev, & Grenier, 2001; Timofeev, Grenier, & Steriade, 2001) of all neocortical neurons (Steriade, Nuñez, & Amzica, 1993a, 1993b; Steriade et al., 2001; Timofeev & Steriade, 1996). The slow oscillations consist of up-states and down-states. Neurons undergo sustained firing during active up-states, whereas down-states are associated with neuronal silence (Amzica & Steriade, 1995; Steriade, Nuñez, et al., 1993a). In the scalp EEG, up-states are reflected in positive deflections of the slow wave, while down-states result in negative slow wave deflections (Amzica & Steriade, 1998; Riedner et al., 2007; Vyazovskiy et al., 2009). Consequently, after neuronal silence, neurons start firing again during negative-to-positive transitions, whereas positive-to-negative transitions are associated with silencing (Riedner et al., 2007; Vyazovskiy et al., 2009).

Interestingly, the degree of neuronal synchronization, which means the conjoint onset of firing and silencing of neural populations, is reflected in the steepness of slopes of the slow oscillations. It underlies a homeostatic process and thus, fading synchrony of the conjoint firing and silencing over the course of the night leads to shallower slow-wave slopes during

late sleep compared with early sleep (Riedner et al., 2007; Vyazovskiy et al., 2009; Vyazovskiy, Riedner, Cirelli, & Tononi, 2007).

Slow oscillations have been associated with memory formation in humans. After learning a declarative memory task, stimulating the brain with 0.75 Hz, the peak frequency of slow oscillations resulted in an enhancement of post-sleep performance compared with a control condition where a stimulation of 5 Hz was applied (Marshall, Helgadóttir, Mölle, & Born, 2006).

Sharp wave-ripples

Hippocampal sharp wave-ripples are fast oscillatory events between 100 to 300 Hz (Mölle, Yeshenko, Marshall, Sara, & Born, 2006) and consist of two oscillatory events: sharp waves and superimposed ripples. Sharp waves are fast oscillations generated by fast bursts of electric activity in pyramidal cells of the hippocampal region cornus ammonis 3 (CA3) that exert a massive depolarization of the hippocampal CA1 region (Csicsvari, Hirase, Mamiya, & Buzsáki, 2000). Interconnectivity between CA3 and CA1 lead to the entrainment of fast bursts of 150 to 250 Hz in the region CA1, also termed "ripples" (Buzsáki, Horvath, Urioste, Hetke, & Wise, 1992; Girardeau & Zugaro, 2011; Ylinen et al., 1995).

Sharp waves typically occur during SWS, but are also present during non-exploratory wakefulness in rats (Buzsáki, 1986). Ripples were associated with compressed replay of waking activity in hippocampal neurons in the same order during sleep (Lee & Wilson, 2002; Nádasdy, Hirase, Czurkó, Csicsvari, & Buzsáki, 1999; Skaggs & McNaughton, 1996), and in the reverse order in wakefulness after exploratory behavior (Csicsvari, O'Neill, Allen, & Senior, 2007; Diba & Buzsáki, 2007; Foster & Wilson, 2006; Karlsson & Frank, 2009).

Besides their role in hippocampal replay of previously learned information, sharp wave-ripples were further associated with memory consolidation because of their time-locked occurrence with slow oscillations up-states. This was evidenced by the finding that sharp wave-ripple events in rats increased during slow oscillation up-states, and decrease during slow oscillation down-states. Importantly, slow oscillations always preceded sharp wave-ripples, demonstrating a synchronizing top-down neocortical-hippocampal influence (Mölle et al., 2006).

Spindles

Thalamo-cortical spindles are oscillatory events visible in the EEG by waxing and waning bursts of activity for 0.5 to 2 s with a frequency between 11 and 16 Hz occurring mainly

during sleep stage N2, but also during N3 (Iber et al., 2007). Spindles are generated in the nucleus reticularis in the thalamus, by interaction between inhibitory GABA cells and excitatory thalamo-cortical neurons. The last transfer spindles over the cortex (Steriade, McCormick, & Sejnowski, 1993; von Krosigk, Bal, & McCormick, 1993) by a massive influx of calcium into pyramidal cells (Sejnowski & Destexhe, 2000). Spindle power increased during the course of nocturnal sleep (Aeschbach & Borbély, 1993; De Gennaro, Ferrara, & Bertini, 2000).

Spindles can be subdivided according to their frequencies in slow spindles (~11 to 13 Hz) and fast spindles (~13 to 15 Hz) (Anderer et al., 2001; De Gennaro & Ferrara, 2003; Mölle & Born, 2011). Concerning topography in the scalp EEG, slow spindles show the highest signal over frontal areas, whereas fast spindle are concentrated at centro-parietal sites (De Gennaro & Ferrara, 2003). EEG and MEG recordings could identify sources of slow spindles in prefrontal areas, in contrast to fast spindles which were found to be located in the precuneus (Anderer et al., 2001; Manshanden, De Munck, Simon, & Lopes da Silva, 2002). Differences between these two types of spindles with regard to involved brain areas have also been demonstrated by functional magnetic resonance imaging (fMRI). Besides common brain regions like the thalamus, superior temporal gyri, and insula cortices, slow spindles are associated with enhanced activation of the superior frontal gyrus, whereas fast spindles are associated with higher activity in the medial frontal cortex and areas involved in sensorimotor processing (Schabus et al., 2007).

Mainly fast spindles have been robustly associated with post-learning sleep retention of declarative memories in many studies, as demonstrated by positive correlations between declarative memory performance and fast spindle density, calculated by the number of fast spindles per minute, (Cox, Hofman, & Talamini, 2012; Gais, Mölle, Helms, & Born, 2002), fast spindle power (Ruch et al., 2012; Schabus et al., 2004), and the number of fast spindles (Clemens, Fabó, & Halász, 2005; Saletin, Goldstein, & Walker, 2011). Moreover, a study using combined EEG and fMRI recordings could show that hippocampal and neocortical activation during post-learning sleep was coupled with spindles. Additionally, the strength of the activation correlated with the spindle amplitude (Bergmann, Mölle, Diedrichs, Born, & Siebner, 2012). These results suggest an important role of spindles in the reorganization of information form the hippocampus to the neocortex.

After the presentation of sleep and unique oscillatory sleep events that have been implicated in memory consolidation, the next paragraph will focus on the interaction between sleep and memory. First, theories for sleep-dependent declarative memory consolidation will

be introduced and the interplay between slow oscillations, sharp wave-ripples and spindles will be explained. Thereafter, the importance of sleep for emotional processing and for extinction memory processing will be stretched.

1.3 Interaction of sleep and memory

Although the exact function of sleep is still partly unknown, several researchers argue that it is critically involved in memory consolidation (Born et al., 2006; Diekelmann & Born, 2010; Rasch & Born, 2013). Already at the end of the 19th century, the memory researcher Hermann Ebbinghaus revealed an impact of sleep on memory processing: As the first, he provided empirical evidence that forgetting of previously learned material is reduced after sleep (Ebbinghaus, 1885). As early as 1924, Jenkins and Dallenbach conducted the first experimental study which systematically examined in a controlled way the influence of sleep on retention after learning. They could show that sleep compared with an equivalent interval of wakefulness led to better memory for nonsense syllables, and interpreted the effect as a result of passive sheltering against interference after learning during sleep (Jenkins & Dallenbach, 1924). This was the impetus for sleep and memory research and since then, numerous studies investigated the role of sleep in memory processing (e.g. Barrett & Ekstrand, 1972; Ekstrand, 1967; Fowler, Sullivan, & Ekstrand, 1973; Newman, 1939; Plihal & Born, 1997, 1999; Richardson & Gough, 1963). Most of these studies found a beneficial effect of sleep on declarative memory consolidation, and some even found hints to a more active role of sleep in memory processing (for a review see Diekelmann & Born, 2010), in contrast to Jenkins' and Dallenbach's suggestion. The following paragraphs will review experimental evidence supporting the active role of sleep in memory processing and the most important theories about the mechanisms of this process. Thereafter, the role of sleep in emotional memory processing will be described.

1.3.1 Sleep-dependent memory consolidation

As already described above, especially in the beginning of sleep and memory research the beneficial effect of sleep on memory was explained as a result of the passive protection of newly acquired, labile memory traces from weakening interference (Jenkins & Dallenbach, 1924). Thus, sleep was seen as a time window during which the new memory trace is not disturbed, but which does not provide any additional benefit for the consolidation process. In

contrast to this point of view, recent studies assume an active role of sleep in memory consolidation. According to this approach, unique parameters occurring during sleep are associated with beneficial post-sleep memory, because they are considered to facilitate system consolidation by enabling a neocortical-hippocampal dialogue and thus a reorganization of memories (Buzsáki, 1989; Mölle & Born, 2011).

The active role of sleep in memory consolidation was first evidenced by studies showing that the same amount of sleep had different impacts on non-declarative and declarative memory consolidation depending on the half of the night: the early SWS-rich half or the late, REM sleep-rich half (Plihal & Born, 1997, 1999). Moreover, animal studies identified a replay of patterns of previously learned information in hippocampal neurons during SWS (O'Neill, Senior, & Csicsvari, 2006; Wilson & McNaughton, 1994). As previously mentioned, in particular slow oscillations (Marshall et al., 2006), spindles (Bergmann et al., 2012; Clemens et al., 2005; Cox et al., 2012; Gais et al., 2002; Ruch et al., 2012; Saletin et al., 2011; Schabus et al., 2004), and sharp wave-ripples (Lee & Wilson, 2002; Nádasdy et al., 1999; Skaggs & McNaughton, 1996) during sleep have been associated with post-learning memory consolidation.

Different hypotheses were stated recently that account for the active role of sleep in memory consolidation and that try to explain its underlying mechanisms. These assumptions, namely the "dual process hypothesis", the "sequential hypothesis", and the "active system consolidation hypothesis", are presented in detail in the following.

The dual process hypothesis

According to the two stages or dual process hypothesis, the consolidation of information processed by different memory systems benefits from different sleep stages. This was first explored by an elegant approach based on the different distributions of the sleep stages during the course of a night: the so-called night-half paradigm. In this paradigm, learning and retrieval of memory are carried out before and after the early, SWS-rich, or the late, REM sleep-rich half of the night. The first study using this paradigm found a beneficial effect of the first night half on declarative word pair association learning compared with sleep during the second night half or daytime wakefulness (Yaroush, Sullivan, & Ekstrand, 1971). A follow-up experiment of the same research group with a refined paradigm controlling for circadian influences by matching the time of the wake control retention interval to the sleep groups could confirm the beneficial role of the SWS-rich first half of sleep on declarative memory (Barrett & Ekstrand, 1972). Other experiments using the night-half paradigm also replicated

the facilitation of declarative memory consolidation by the SWS-rich first part of the night using word pair (Fowler et al., 1973; Plihal & Born, 1997) or spatial location learning (Plihal & Born, 1999). Additionally, evidence for a beneficial effect of the second half of the night for non-declarative, procedural memory was confirmed using tasks to examine skill memory (Plihal & Born, 1997) and priming (Plihal & Born, 1999; Wagner, Hallschmid, Verleger, & Born, 2003). These studies suggest the importance of SWS in declarative memory formation, whereas REM sleep rather seems to influence non-declarative memory.

However, there are also findings that are not consistent with the dual process hypothesis. Sleep stage N2 also plays a critical role in sleep-dependent memory consolidation, as evidenced by studies that associate this sleep stage with simple motor learning tasks (Fogel, Smith, & Cote, 2007; Smith & MacNeill, 1994). As N2 sleep is equally contributed over the two halves of nocturnal sleep, the dual process hypothesis does not explain this finding. Furthermore, SWS seems to facilitate the consolidation of non-declarative procedural memory by SWS (Huber, Ghilardi, Massimini, & Tononi, 2004), and several studies reported a beneficial effect of REM sleep on declarative memory consolidation (Fogel et al., 2007; Gais, Plihal, Wagner, & Born, 2000). Besides the possibility that memory tasks do not exclusively involve and measure the target memory type, another explanation for the latter findings will be presented next. However, this so-termed "sequential hypothesis" does also not account for the findings concerning N2 sleep.

The sequential hypothesis

Compared with the assumption of the dual process hypothesis that sleep stages differentially impact different memory systems, the sequential hypothesis assumes that the succession of SWS and REM sleep within one sleep cycle is critical, indicating that these sleep stages rather interact than differentially impact memory. According to the hypothesis, non-adaptive memory contents are first weakened during SWS. In a second step, REM sleep causes an integration of the adaptive memory into already existing memory networks (Ambrosini & Giuditta, 2001; Giuditta, 2014; Giuditta et al., 1995). Studies investigating sleep including full, synchronized SWS-REM cycles compared with awakenings after SWS provide evidence for the sequential hypothesis. For example, the consolidation of memory for an avoidance task correlated positively with the number of synchronized SWS-REM episodes (Ambrosini & Giuditta, 2001; Ambrosini et al., 1993; Langella, Colarieti, Ambrosini, & Giuditta, 1992). Also in humans, sleep containing synchronized SWS-REM cycles was beneficial for declarative word pair memory compared with disrupted SWS-REM succession (Ficca,

Lombardo, Rossi, & Salzarulo, 2000). Furthermore, insight into complex linguistic rules profited from the product of SWS and REM sleep durations (Batterink, Oudiette, Reber, & Paller, 2014), and visual-procedural non-declarative memory was only improved after the succession of SWS and REM sleep in a nap compared with no-REM sleep naps (Mednick, Nakayama, & Stickgold, 2003). Interestingly, evidence for complementary roles of REM sleep and SWS were also found for emotional memory consolidation during post-sleep recognition of emotional and neutral images. Hereby, the amount of nocturnal SWS predicted better memory for negative pictures and less hippocampal activity in response to these negative stimuli. The amount of REM sleep, however, predicted increased hippocampal-neocortical connectivity for negative pictures (Cairney, Durrant, Power, & Lewis, 2014). Despite the support of the sequential hypothesis that arises of these results, it is rarely tested in sleep studies and further empirical evidence is needed.

Although the sequential hypothesis and the dual process hypothesis both assume an active role of sleep in memory consolidation, these two hypotheses do not provide insight in the neural processes of sleep-dependent memory consolidation. One approach that further explains the interaction of certain sleep parameters on a neural basis is the active system consolidation hypothesis, as explained in the following section.

The active system consolidation hypothesis

The active system consolidation hypothesis combines assumptions of the sequential and the dual process hypotheses but rather concentrates on neural mechanisms underlying the sleep-dependent memory effect than simply linking sleep stages to memory or memory types. This hypothesis specifies the process of system consolidation and builds up on two central mechanisms: the two-stage model of memory consolidation (Marr, 1971) and spontaneous hippocampal replay of previously learned information during SWS (Wilson & McNaughton, 1994), which will be outlined in detail in the following sections before presenting the actual hypothesis.

The standard two-stage model of memory consolidation, as described earlier in the memory part of the introduction (Marr, 1971), is the first part of the active system consolidation hypothesis. The model assumes the coexistence of a fast learning short-term storage in the hippocampus and a slow learning long-term storage in the neocortex. New information acquired during encoding in wakefulness is stored in the hippocampus and the neocortex in parallel. The information undergoes reorganization by hippocampal reactivation and becomes progressively hippocampus-independent and integrated in already existing

neocortical networks (see Figure 3 a). Sleep is assumed to play an important role in this system consolidation process (Buzsáki, 1989; Marr, 1971).

First hints for such a hippocampal reactivation of previously learned reactivations during sleep came from a rodent study (Wilson & McNaughton, 1994). Single cell recordings revealed the same pattern of activation in hippocampal neurons during SWS as in wakefulness. Further studies suggested that this replay during sleep occurred in a compressed time scale but in the same order as during encoding in wakefulness (Lee & Wilson, 2002; Nádasdy et al., 1999; Skaggs & McNaughton, 1996). In contrast, such a replay was also observed in a compressed, reversed manner during post-exploratory wakefulness (Csicsvari et al., 2007; Diba & Buzsáki, 2007; Foster & Wilson, 2006; Karlsson & Frank, 2009). The underlying electrophysiological oscillation during this replay is a high-frequency burst of activity, termed "sharp wave-ripple".

To conclude a causal role of these covert memory reactivations during sleep, this mechanism was elegantly biased in a paradigm by applying an olfactory background cue during learning and re-presenting it during post-learning SWS. This leads to an incorporation of the context background cue into the new memory trace, and the presentation of this background cue reactivates memory. Such a paradigm resulted in better memory and evidenced the causal role of sleep in memory consolidation (Rasch, Büchel, Gais, & Born, 2007). This paradigm will be referred to in the following as "targeted memory reactivation" (TMR).

These two mechanisms of system consolidation and hippocampal replay during sleep are combined in the active system consolidation hypothesis (Mölle & Born, 2011). This approach assumes an interaction between cortical slow oscillations, thalamo-cortical spindles, and hippocampal sharp wave-ripples. More precisely, slow oscillations with a peak frequency of 0.75 Hz synchronize thalamo-cortical spindles, bursts of 11 to 15 Hz, and hippocampal sharp wave-ripples with frequencies from 100 to 300 Hz (Mölle & Born, 2011). Importantly, as already described in the sleep section, the up-states of the slow oscillations are associated with synchronous firing of cortical neurons, whereas the down-states entrain neural silence (Riedner et al., 2007; Vyazovskiy et al., 2009). Thus, the orchestrating top-down influence of the slow oscillations appears only during the up-states. It leads to spindle-ripple events by nesting hippocampal sharp wave-ripples into the repeated troughs of the spindles. Because the spindle-ripple events occur with a much faster frequency than the slow oscillations, hippocampal information reaches the neocortex during the up-state, enabling a bottom-up transfer of hippocampal information to the neocortex (see Figure 3 b). Such a dialogue

between hippocampus and neocortex is suppressed during wakefulness due to high levels of acetylcholine, a neurotransmitter which exerts an inhibiting influence on hippocampo-cortical projections. Since acetylcholine is very low-concentrated during SWS, these pathways can be used to transfer information (Hasselmo, 1999). With reference to the sequential hypothesis, this model further suggests that synaptic consolidation after memory redistribution might take place during subsequent REM sleep (Diekelmann & Born, 2010; Mölle & Born, 2011).

To sum up, the model and mechanisms underlying the active system consolidation hypothesis are acknowledged and were evidenced by several animal studies. The verification of this model in human studies based on EEG – since cortical cell recordings are ethically questionable and not an option in healthy humans – still needs to be done.

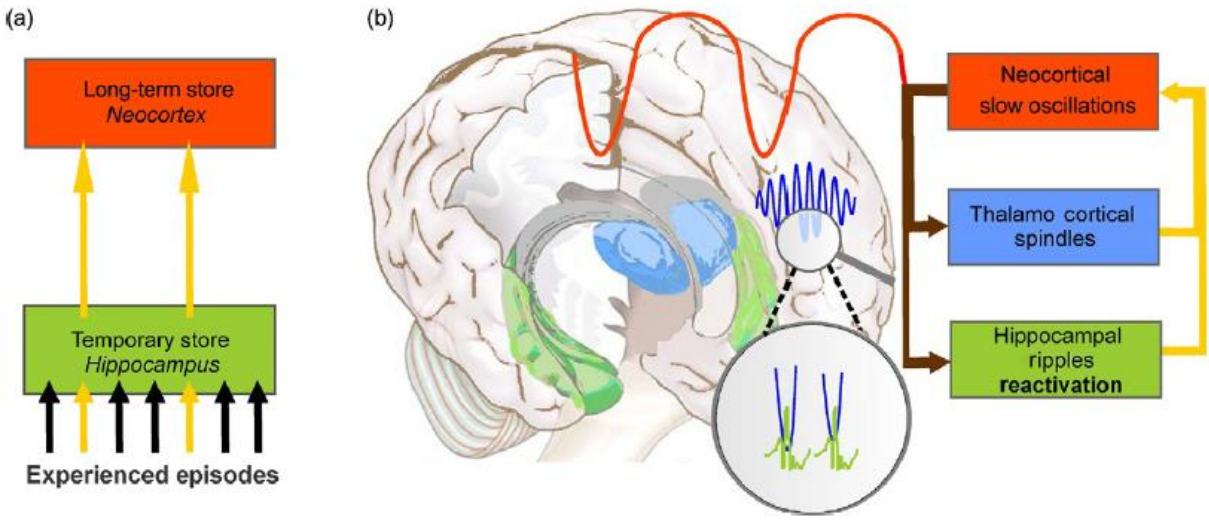

Figure 3. Active system consolidation process.
(a) Newly acquired, declarative memories are temporarily stored in the hippocampus after encoding. During system consolidation, they are reorganized to the neocortex and become hippocampal-independent. (b) As neural mechanisms behind this process, an interaction between slow oscillations, spindles, and sharp wave-ripples is assumed. From Mölle & Born (2011).

1.3.2 Olfactory targeted memory reactivation

As already mentioned, odors have been used in design of the first human TMR study to cue previously learned memory (Rasch et al., 2007), and the memory-enhancing effect due to this paradigm has been successfully replicated (Diekelmann, Büchel, Born, & Rasch, 2011). Odors were used as background cues because they do not wake when presented during deeper sleep stages (Carskadon & Herz, 2004). However, the TMR effect has also been replicated

with auditory background stimuli (Antony et al., 2012; Oudiette, Antony, Creery, & Paller, 2013; Rudoy, Voss, Westerberg, & Paller, 2009).

The effectiveness of olfactory TMR cues during sleep still needs to be further investigated. For example, since the control condition in these designs always included an odorless vehicle and no other odorants, it could hypothetically be that only an unspecific activation of the olfactory system during sleep is sufficient to activate the hippocampal information associated with the memory. Although such an assumption was rejected for auditory cues by successfully targeting a big number of individual sounds (Rudoy et al., 2009), evidence for such a TMR specificity of odors is not investigated.

Furthermore, an assumption about the effectiveness of odors as TMR stimuli is based on the unique characteristics of the olfactory system compared with other sensory modalities: It holds direct projections between the olfactory cortex and regions implicated in memory, such as the hippocampus and the amygdala by bypassing the thalamus (Gottfried, 2006). Despite piriform cortex hyporesponsiveness to olfactory stimulation during sleep compared with wakefulness (Barnes, Chapuis, Chaudhury, & Wilson, 2011), odors are excellent TMR stimuli (Diekelmann et al., 2011; Rasch et al., 2007). This leads to questions about the underlying neural mechanisms of olfactory processing during sleep. Until now, no study further investigated this question.

1.3.3 Sleep and emotional memory

Emotional memories are remembered better than neutral ones (Cahill & McGaugh, 1998; Holland & Lewis, 2007; McGaugh, 2006). Besides the adaptive value of emotional memory, highly arousing and stressful events may lead to intrusive and long-lasting traumatic memories (Leskin, Woodward, Young, & Sheikh, 2002), repeated nightmares (Wittmann, Zehnder, Schredl, Jenni, & Landolt, 2010), affective disorders (Hammen, 2005), and anxiety disorders (Faravelli, 1985; McLaughlin & Hatzenbuehler, 2009). Evidence accumulated that sleep, and especially REM sleep, has an impact on emotional processing and emotional memory consolidation. The next sections will review evidence for a role of sleep, first in emotional memory consolidation, and second in emotional processing.

Sleep and emotional memory consolidation

In contrast to the knowledge about the role of sleep in the consolidation of neutral declarative memories and the clear evidence for its underlying mechanisms, the impact of sleep on

emotional memory processing is rather scarce and ambiguous. Yet, it is widely assumed that sleep also plays a critical role in emotional memory processing. For example, the consolidation of memory for emotionally learned associations (Menz et al., 2013), emotional texts (Wagner, Hallschmid, Rasch, & Born, 2006), and emotional pictures or scenes (Baran et al., 2012; Hu et al., 2006; Payne, Chambers, & Kensinger, 2012) profits from sleep compared with sleep deprivation or day-wakefulness. Although the majority of studies found a sleep-dependent enhancement of emotional memory consolidation, two studies contradict these results. These studies found superior retrieval of previously learned negative (Sterpenich et al., 2007) and emotional (Atienza & Cantero, 2008) pictures compared with neutral pictures for participants that underwent nocturnal sleep deprivation compared with a normal night of sleep.

In addition to the majority of findings that associate emotional memory consolidation with sleep, particularly REM sleep seems to be the key player in emotional memory consolidation, although literature is also ambiguous here. Already Freud suggested an influence of dream sleep, today termed REM sleep, on reprocessing emotional events (Freud, 1900). The critical involvement of REM sleep in emotional memory consolidation was indeed inferred from several findings. First, a study using positron emission tomography (PET) found increased regional blood flow during REM sleep in amygdaloid regions, which are usually involved in emotional processing (Maquet et al., 1996). Second, the beneficial effect of REM sleep on post-sleep emotional memory performance correlated with time spent in REM sleep (Nishida, Pearsall, Buckner, & Walker, 2009; Payne et al., 2012) or with the amount of theta power during REM sleep (Nishida et al., 2009). Third, empirical evidence also comes from studies investigating the differential effects of the early, SWS-rich and the late, REM sleep-rich night half on emotional memory. For example, memory for emotional texts was stronger after the second, REM-rich half of the night (Wagner et al., 2001), and emotional pictures were better remembered after late sleep (Groch, Wilhelm, Diekelmann, & Born, 2013; Groch, Zinke, Wilhelm, & Born, 2014; Wagner, Fischer, & Born, 2002). Fourth, experimental paradigms testing the importance for REM sleep in emotional memory tasks by depriving participants of REM sleep found that memory for emotionally-laden stories is impaired when REM sleep is lacking (Greenberg, Pearlman, Schwartz, & Grossman, 1983). As a last point, the implication of REM sleep in emotional processing can be derived from the fact that REM sleep is dysregulated and disinhibited as comorbidity in disorders affecting emotional processing, such as depression (Tsuno, Besset, & Ritchie, 2005) and post-traumatic stress disorder (PTSD) (Germain, 2013; Mellman & Hipolito, 2006; Spoormaker & Montgomery,

2008). This disinhibition manifests itself for example in decreased REM sleep latency and an increased amount of REM sleep in early sleep in patients with major depressive disorders (Tsuno et al., 2005).

Furthermore, concerning affective disorders and sleep, an interesting observation consists in the finding that the return to normal of dysregulated sleep architecture is associated with a diminished risk of relapse (Ohayon, 2007) and successful psychological treatments of depression decreased REM density (Buysse, Frank, Lowe, Cherry, & Kupfer, 1997; Nofzinger et al., 1994). Additionally, sleep deprivation in depressive patients is a well-acknowledged treatment. It results in a short-lasting relief of depression symptoms, leading for example to normalized mood, possibly due to an interruption of dysfunctional sleep. In contrast, sleep deprivation in healthy participants resulted in dysfunctional emotional encoding, by biased encoding of negative (Yoo, Hu, Gujar, Jolesz, & Walker, 2007) as well as positive (Gujar, Yoo, Hu, & Walker, 2011) stimuli compared with normal sleep. This could be explained by the important role of healthy sleep in emotional processing, as evidenced in the following section. In depressed patients, however, dysfunctional sleep could play an important role in dysfunctional emotional processing and emotional memory processing, and disrupting abnormal sleep could return normal emotional processing. Therefore, knowledge about differences in brain activity between wake, normal sleep and sleep deprivation could help to further specify the role of sleep in emotional processing and in affective disorders.

Although the majority of findings points to a sleep-dependent increase in emotional memory consolidation, the critical sleep stage involved in this consolidation is still unclear. Even though there is vast evidence for a critical role of REM sleep in emotional memory consolidation, newer studies also underline an important involvement of SWS and sleep spindles in emotional memory consolidation (Cairney, Durrant, Hulleman, & Lewis, 2014; Cairney, Durrant, Jackson, & Lewis, 2014; Cairney, Durrant, Power, et al., 2014). An important aspect that could account for some of these differences is the nature of the learning task, namely its proportions of declarative, hippocampus-dependent compared with non-declarative, amygdala-dependent contents and the aversion of the negative stimuli in these experiments, for example electric shocks compared with negative pictures. Another possible explanation of these different findings is the notion that REM sleep and SWS complement one another in consolidation of emotional memory during sleep. A recent study found such complementary roles for SWS and REM sleep in emotional consolidation. The duration of nocturnal SWS predicted superior memory for and less hippocampal activity to these negative stimuli, whereas the duration of REM sleep was correlated with increased hippocampal-

neocortical connectivity during recognition of negative pictures (Cairney, Durrant, Power, et al., 2014).

Sleep and emotional processing

Besides the preferential consolidation of memory for emotional over neutral events, sleep, and in particular REM sleep, also has an impact on emotional processing with regard to the intensity of the emotion. This manifests itself in altered intensity of emotional stimuli after sleep compared with sleep-deprived or normal wake control groups. This intensity is also referred to as emotional reactivity. Subjective measures of emotional reactivity are usually assessed by arousal and valence ratings in response to emotional stimuli, whereas objective measures are first the activation of brain regions involved in emotional processing, typically the amygdala, and second psychophysiological parameters such as electrodermal activity.

The importance of a full night of sleep compared with sleep deprivation on emotional processing was already pronounced at encoding of emotional and neutral stimuli. Two studies demonstrated that sleep-deprived healthy participants showed an emotional bias during encoding emotional and neutral stimuli, in a way that negative (Yoo et al., 2007) as well as positive (Gujar, Yoo, et al., 2011) pictures elicited higher reactivity in the sleep-deprived group, shown by increased amygdala activation during encoding of these stimuli.

Additionally to effects of sleep on emotional reactivity during encoding, sleep after learning likewise impacts emotional reactivity during recall. However, results here are ambiguous: The majority of studies found an enhancing or preserving effect of sleep on emotional reactivity, whereas only few studies found diminished emotional reactivity associated with sleep. On the one hand, nocturnal sleep in general compared with sleep deprivation increased amygdala activation to negative compared with neutral pictures (Sterpenich et al., 2009) and to negatively conditioned associations (Menz et al., 2013). Furthermore, in contrast to a day of wakefulness, a night of sleep enhanced the connectivity between amygdala and hippocampus for correctly recognized, negative objects (Payne & Kensinger, 2011). As for emotional memory consolidation, there is also evidence for emotional processing that superior emotional reactivity is associated with REM sleep. First, when sleep after learning consisted of REM-rich, late sleep compared with SWS-rich, early sleep, valence ratings to negative pictures were increased (Wagner et al., 2002), and valence and arousal ratings to negative pictures were preserved (Groch et al., 2013). Second, the duration of REM sleep predicted the increase in skin conductance responses (SCR) to picture-symbol associations previously paired with a shock (Menz et al., 2013), the increase in SCR

and less reduction in electromyogram (EMG) responses to a negative compared with a neutral film (Werner, Schabus, Blechert, Kolodyazhniy, & Wilhelm, 2015), and the preservation of arousal and valence ratings to negative pictures (Baran et al., 2012). Third, a post-hoc group assignment in a nap study to participants whose nap contained REM sleep compared with the absence of REM sleep revealed that the duration of REM predicted less decrease of SCR (Pace-Schott et al., 2011). Fourth, and last, a study that used nocturnal REM sleep deprivation compared with normal, REM sleep-containing sleep found less decrease of arousal ratings in the REM group (Lara-Carrasco, Nielsen, Solomonova, Levrier, & Popova, 2009). On the other hand, however, there are also studies that cannot confirm a sleep-dependent reactivity-enhancing effect, but rather found a decrease in emotional reactivity after sleep. For example, post-sleep retrieval of emotional pictures revealed larger emotional reactivity after sleep deprivation compared with nocturnal sleep, expressed in higher amygdala activation to negative pictures (Sterpenich et al., 2007). In accordance with this finding, nocturnal sleep compared with daytime wake decreased amygdala activation and emotional intensity in response to negative pictures (van der Helm et al., 2011). In a face recognition nap study with a wake group and post-hoc assignments of the sleep participants to a REM sleep-containing and a REM sleep-absent nap group, lowered intensity ratings to aversive faces were only revealed after REM sleep (Gujar, McDonald, Nishida, & Walker, 2011). Furthermore, after learning emotional pictures, selective nocturnal REM sleep deprivation compared with NREM deprivation lead to increased self-reported reactivity when REM sleep was absent and to an overall decrease in fMRI brain activity in the group that experienced post-learning REM sleep (Rosales-Lagarde et al., 2012).

To sum up, even though results are ambiguous, the majority of findings underline the importance of sleep, and in particular REM sleep, in emotional consolidation and emotional processing. However, the sleep stage and sleep parameters associated with emotional memory consolidation are still unclear, since newer studies found a beneficial effect of spindles, spindles in SWS, or of a SWS-REM sleep interaction on emotional memory consolidation. For emotional processing during sleep, REM sleep seems to be the key player, also evidenced by its critical role in disorders associated with dysfunctional affective processing. A model that accounts for these findings and introduces a theoretical assumption about the underlying neural mechanisms will be presented next.

Sleep to forget and sleep to remember

The only theoretical account so far that suggests an underlying mechanism for emotional memory processing during sleep is termed the "sleep to forget and sleep to remember" (SFSR) model (Walker & van der Helm, 2009; Walker, 2009, 2010). According to this theory, emotional memories consist of a core memory about the event "wrapped" with an emotional tone. During post-learning REM sleep, the emotional memory is reprocessed in a special way: The affective tone of the emotional event is reduced, which leads to a reduction, or forgetting, of the emotional reactivity, while the declarative, informational part of the emotional event undergoes strengthening, which leads to remembering. This process is repeated over several nights, leaving only the neutral information as part of the emotional memory (see Figure 4). As everyday evidence for this theory, the authors describe the well-experienced effect that autobiographical, emotional events can still be retrieved long time after learning, but vivid emotions associated with the emotional memory fade over time (Van Der Helm & Walker, 2011).

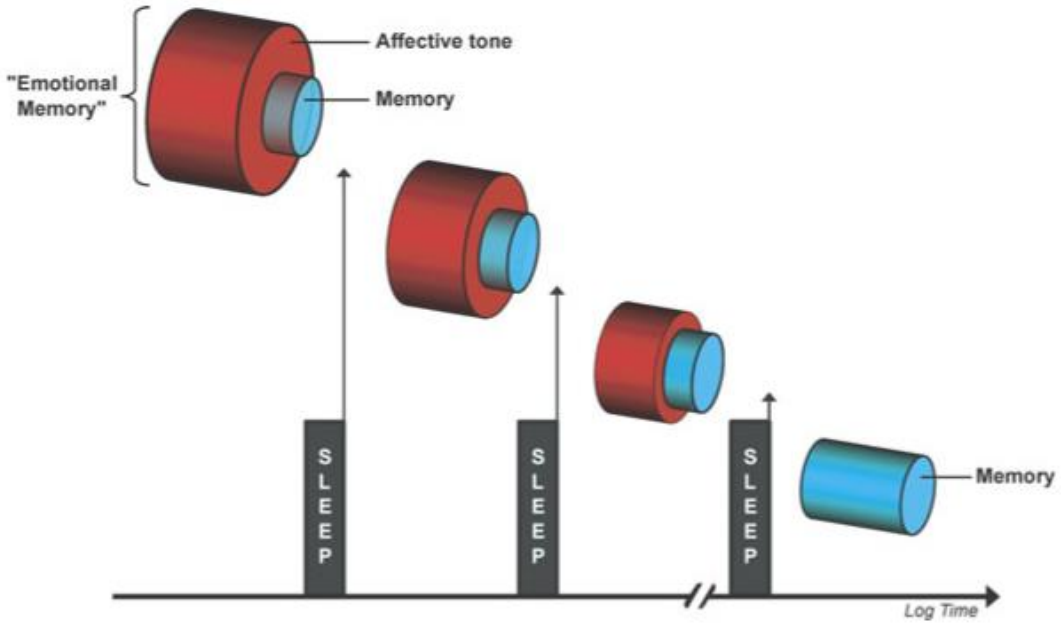

Figure 4. Processing of emotional memory according to the "Sleep to Forget and Sleep to Remember" hypothesis.
An emotional memory consists of an affective tone and a neutral memory for the event. The affective tone of the emotional memory is repeatedly reduced during REM sleep, leaving only the neutral, informational content of the memory after a while. From Walker (2009).

On a neural basis, the authors argue that the unique biology of REM sleep contributes to the above described emotional memory processing. The increased activity of limbic and

paralimbic structures during REM sleep could hint to covert reactivation of emotional memories, whereas the absence of a neuromodulator associated with high stress levels and anxiety disorders, namely noradrenalin, is assumed to "neutralize" the emotional tonus during the covert reactivation (Walker & van der Helm, 2009).

Applying this model to the previously described study results, literature only partly supports the model. As described above, consolidation of the emotional memory seems indeed to profit from REM sleep, whereas emotional reactivity rather seems to be enhanced after REM sleep, instead of reduced. Of note, a small number of findings could confirm a decrease in emotional reactivity. Due to these ambiguities, the SFSR hypothesis needs to be further investigated. One such possible way to further test this model and to determine the critical involvement of REM sleep in emotional memory processing would be by examining post- to pre-learning changes in emotional reactivity and emotional memory consolidation after TMR during REM sleep. The next section describes the few studies that used such an approach. Importantly, they mainly focused on emotional memory consolidation.

Targeted memory reactivation of emotional memory during REM sleep

Early studies in rodents investigated the effect of re-presenting a negatively learned stimulus or a background cue present during aversive Pavlovian conditioning in post-learning REM sleep. For instance, representing CS+ stimuli that were emotionally learned by pairing with an UCS as memory cues during post-learning REM sleep activated hippocampal neurons (Maho & Bloch, 1992) and enhanced emotional memory consolidation as shown by increased avoidance behavior in response to negatively learned stimuli (Hars, Hennevin, & Pasques, 1985), whereas CS+ cueing during SWS impaired emotional memory consolidation (Hars et al., 1985).

Compared with the wide range of human TMR studies testing the role of SWS in declarative memory consolidation, so far there is only one study in humans applying the concept of TMR on emotional memory processing and consolidation, with memory reactivation by CS+ presentation during REM sleep. Surprisingly, this study could not find behavioral effects of TMR on emotional memory consolidation (Sterpenich et al., 2014).

Obviously, there is not only no clear evidence from TMR in human studies that REM sleep is critically involved in emotional memory consolidation, but findings even suggest that NREM sleep also seems to play an important role. For example, NREM sleep is increased after aversive learning, whereas the time spent in REM sleep after emotional learning is decreased (Talamini, Bringmann, Boer, & Hofman, 2013). Moreover, recent studies using

TMR in SWS provide evidence that this sleep stage is critically involved in emotional memory consolidation: In rats, the re-presentation of an odor presented as CS+ during fear conditioning in SWS lead to stronger fear behavior during retrieval testing (Rolls et al., 2013). In humans, after learning picture-location associations under the presence of a related background sound, re-presentation of the sounds during SWS led to increased consolidation, as shown by faster post-sleep reaction times to CS+ stimuli (Cairney, Durrant, Hulleman, et al., 2014). In another study, a background odor was present during aversive conditioning by face-shock pairings. TMR with the background odor during SWS lead to the extinction of these emotional memories. This was paralleled by a continuous reduction of SCRs during sleep, possibly reflecting a habituation to the stimulus, and thus a reduction in emotional reactivity (Hauner et al., 2013). However, all of these studies did not contain a control REM sleep condition.

To sum up, the above reviewed literature about emotional memory consolidation and emotional processing in REM sleep and empirical evidence for the SFSR theory are very ambiguous. It still remains an open question which sleep stage is critically involved in the re-processing of emotions and emotional memories, and if this sleep stage has an impact on both, emotional memory consolidation and emotional reactivity.

1.3.4 Sleep and fear extinction memory

In addition to the above mentioned effects on emotional memory processing, recent studies provide evidence that sleep also plays an important role in extinction memory consolidation. As shown in healthy participants who underwent fear conditioning under the presence of background odorants, the re-presentation of these odors during post-conditioning SWS leads to extinction, as demonstrated by decreased skin conductance responses during reactivation in sleep compared with reactivation in wakefulness, by decreased hippocampal activity after sleep, and by a different pattern in brain activation in the amygdala after sleep (Hauner et al., 2013).

Even in a clinical context, the beneficial effect of post-treatment sleep on the newly acquired extinction memory traces learned during psychological behavioral therapy has been shown in spider phobic patients. For example, spider phobic patients who slept after *in sensu* exposure by video showed enhanced retention of fear extinction memory and enhanced generalization compared with patients that remained awake (Pace-Schott et al., 2012). The beneficial effect of sleep in strengthening therapeutic fear extinction memory was further

confirmed by another study that exposed spider phobic patients to spiders in a virtual environment. One week after exposure, participants who napped after the treatment reported less subjective fear while approaching a spider than patients who stayed awake. Moreover, the decreased fear ratings were positively correlated with the percentage of N2 sleep during the nap (Kleim et al., 2013). These results suggest that sleep, and especially NREM sleep, benefits the consolidation of extinction memory. However, until now no study tested if the neural mechanisms underlying this effect on extinction memory of fear patients are similar to those during declarative memory consolidation. An easy way to test this would be by applying TMR during post-treatment sleep.

2 Aims and hypotheses

The aims of the studies were to further define electrophysiological correlates of the mechanisms involved in the active role of sleep in declarative memory processing, as well as the application of the TMR effect to other memory types that have been investigated less frequently or not until now, namely emotional memory and extinction memory. We hypothesized that sleep-dependent memory reactivation results in EEG oscillation changes. Furthermore, we assumed that besides declarative memory consolidation, sleep is also critically involved in emotional memory consolidation, emotional re-processing, and extinction memory consolidation. This should be reflected by superior post-sleep retrieval for targeted memories, and, in the case of emotional processing, altered emotional reactivity towards these memories. The following section presents the specific aims and hypothesizes of the five studies.

The aim of the first study was to investigate if a congruency of olfactory stimuli presented during TMR and learning is needed to induce a memory-enhancing effect, or if a mere activation of the olfactory system during sleep with incongruent odors is sufficient to trigger hippocampal activation. Furthermore, we aimed at determining EEG oscillation changes in response to TMR, and thus involved in memory consolidation, during presentation of the memory-associated olfactory cue. We expected to replicate the beneficial effect of TMR during SWS on declarative memory consolidation with a congruent, but not with an incongruent odor. On the electrophysiological level, congruent olfactory TMR should result in changes in sleep EEG parameters associated with memory processing, such as spindles, slow oscillations, and slow wave activity.

In the second study, we aimed at identifying possible differences between neural correlates involved in olfactory processing during sleep compared with wakefulness, which could account for the robust olfactory TMR effect despite hyporesponsiveness of the piriform cortex during sleep. Therefore, we presented odors that were not associated with memory during sleep and wakefulness in an fMRI scanner. We expected to replicate hyporesponsiveness of the piriform cortex to odors during sleep, whereas other brain structures involved in memory processing, such as the hippocampus and the amygdala, should show how higher activation during sleep compared with wakefulness.

We conducted the third study to determine the role of REM sleep for emotional memory consolidation and reactivity by using TMR. We hypothesized that emotional memory

consolidation is enhanced after TMR in REM sleep, and that emotional reactivity in response to these memories is increased during post-sleep retrieval.

In the fourth study, our aim was to examine if olfactory TMR of *in vivo* exposure-based group therapy success in patients with specific phobia is beneficial for extinction memory. We hypothesized that extinction memory TMR causes specific responses in sleep EEG parameters associated with memory, and that TMR further leads to less fear towards the phobic object and hence supports therapy success.

As a preliminary study that aimed at paving the way for application to a clinical sample, we examined possible differences in resting state brain activity between normal wake and sleep-deprived healthy participants. Such differences could account for beneficial effects of sleep deprivation-therapy in patients with affective disorders. We hypothesized that brain activity differs between rest in sleep and sleep-deprivation, such that typical hyperactivity in depression-related regions and networks is reduced after sleep deprivation.

3 General methods

As a short overview, the next section provides an overview of the methods used to operationalize the above mentioned research questions and applied in the following studies. A more detailed description of the methods is given in the different manuscripts in the following section. First, the different memory tasks used in the manuscripts are described. Second, targeted memory reactivation by presenting previously associated background cues during sleep is explained. Third, the acquisition, processing, and analysis of sleep data are described.

3.1 Memory tasks

In three of the following five studies we investigated the impact of TMR on declarative, emotional, or extinction memory. Therefore, the memory tasks to establish pre-sleep learning are presented in short here before introducing the manuscripts.

3.1.1 Declarative memory

In the first study, we examined the role of sleep in declarative memory consolidation and associated changes in EEG parameters. We experimentally induced declarative, hippocampus-dependent, object-location learning by a task resembling the game "concentration" which consists of 15 card pairs of colored animals and everyday objects. Participants see the cards as gray squares ordered in a checkerboard-like fashion on a computer screen ("the back of the cards").

The learning task started by presentation of one card of each card pair alone, followed by the simultaneous presentation of the second card of the pair. All card pairs of the set were presented twice. Thereafter, immediate recall took place by presenting one card and the participant had to identify the location of the second card. Independent of the correctness of the trial, visual feedback on the performance was given by displaying the correct location in order to enable re-encoding of the correct card pair location. This procedure was repeated until 60% of the responses were correct. After a card pair was presented, the locations on the screen were replaced again by gray squares to keep guessing probability constant throughout the task. In order to associate the newly learned memory with a cue, a background odor was presented, starting with the presentation of the first card and ending after both cards were

replaced again. During post-sleep retrieval of declarative memory the next morning, the same procedure as during learning was used, but without odor presentation this time.

To analyze differences between the different sleep interventions on memory consolidation, the percentage of the correctly remembered card locations at retrieval was computed, with memory performance in the last pre-sleep run set to 100%.

3.1.2 Emotional memory

In the third study, we investigated the impact of sleep on emotional memory consolidation. We induced emotional learning with Pavlovian aversive conditioning by establishing an association between neutral conditioned stimuli (CS) and a negative unconditioned stimulus (UCS). As a result of the repeated pairing of the CS (CS+) with the UCS, the neutral stimuli became negatively valenced, and thus emotional. As control condition, other CS (CS-) were never paired with the UCS, and thus kept their neutral valence.

As initially neutral CS, we took eight normalized sounds of the International Affective Digitized Sound (IADS) system. These sounds have durations of 6 s. Aversive learning was created by presenting a negative odor (4-methyl pentanoic acid) with half of the sounds after 3 s (CS+). As a control condition, the odorless vehicle was presented to the other sounds after 3 s (CS-). All CS+ and CS- were presented 10 times, and the reinforcement rate was 80%, which means the CS+ was presented 8 times with the odor and two times with the odorless vehicle.

As measure of emotional learning, we used ratings of UCS expectancy. In a first run, all of the eight odors were presented with odor or vehicle pairing, to habituate participants to the stimuli. After habituation, emotional learning started. In ten runs, each of the sounds was presented for 1 s, and participants were asked to rate their odor expectancy (“How likely will the odor be presented at the end of this sound?”) from one (“very unlikely”) to nine (“very likely”). A rating of five indicated uncertainty (“I do not know”). When they responded by key press, the odor was released three seconds after the sound onset and was perceivable at 4.5 s after sound onset. If they failed to answer within 2 s, they received feedback that they were too slow, the odor was not released, and the trial was re-started. The intertrial interval was randomized between 13 and 17 s.

Recall took place two days later in the evening and the procedure was the same as during learning, but without the presentation of the odor this time.

Emotional memory consolidation was analyzed by computing the difference between expectancy ratings to CS+ and CS- stimuli at the end of learning and the beginning of recall. The reaction times of these ratings were also analyzed the same way. For learning, we took the last half of the conditioning trials, whereas for recall, we only considered the first trial of each of the eight sounds for this analysis, to avoid extinction effects.

Additionally, in order to determine changes in subjective emotional intensity attributed to the different sounds, emotional reactivity was assessed by four valence and arousal ratings to each of the sounds. Valence and arousal were included in the task and rated immediately before and after learning and recall. Importantly, to avoid extinction when the CS+ stimuli were presented without the UCS, we explicitly instructed the participants that this run will only contain the sounds without odor pairings.

3.1.3 Extinction memory

In the fourth study, we investigated the role of sleep for fear extinction in patients. Therefore, we extinguished fear in anxiety patients: We applied two sessions of *in vivo* exposure-based group therapy to spider phobic patients. In short, in the first session, the patients first underwent a theoretical session including psychoeducation about spider phobia and its treatment with exposure therapy, fear circuits, avoidance behavior, and group rules. A practical session followed, during which patients first looked at the spider in a wine glass and then touched the spider with a pen. In the second therapy session one week later, the practical session was continued and patients additionally touched the spider with a finger, caught it with a glass, and let it walk over their hand. The therapy sessions were guided by an experienced psychotherapist.

At the end of both sessions, positive feedback rounds took place, during which each participant verbalized their subjective feeling of therapy success and self-efficacy. We presented an odor as background cue during the feedback round of the first session in order to establish an association between this odor and extinction learning.

We assessed the fear of spiders by questionnaires concerning symptoms and intensity of subjective fear of spiders, the ability to approach a spider, and subjective arousal and SCRs to spider pictures. To investigate an impact of sleep on extinction memory, we compared the values of the fear of spider parameters between groups at the beginning, pre-sleep at the end of the first session, post-sleep at the end of the first session, and at the beginning of the second session one week later. Changes in extinction memory were calculated by subtracting the

values between these time points (absolute differences) and as percentage, with the very first measure set as 100% (for the post-sleep interval), or the last measure of session one as 100% (for the first assessment during session 2) and compared between groups.

3.2 Targeted memory reactivation during sleep

The presentation of a stimulus in sleep that was previously associated with pre-sleep learning is termed "targeted memory reactivation" (TMR). TMR is the method of choice to examine if a certain sleep stage is critically involved in memory processing. In order to enable TMR it is necessary to experimentally establish an association between a newly acquired memory trace and a background stimulus. In one of three TMR studies presented in the next section, we use negatively learned sounds as memory cues during sleep, since they have been proven to be effective TMR cues (Antony et al., 2012; Oudiette, Antony, Creery, & Paller, 2013; Rudoy, Voss, Westerberg, & Paller, 2009). In two of our three sleep TMR studies, we used olfactory stimuli as background cues for the following reasons: First, background odor cueing is a well-established, potent method for TMR during sleep (Diekelmann et al., 2011; Rasch et al., 2007). One aspect that might explain the great efficacy of odors to reactivate memories during post-learning sleep are the anatomical features of the olfactory system, such as direct projections of the olfactory cortex to the hippocampus and the amygdala, without bypassing the thalamus (Gottfried, 2006). Second, non-trigeminal odors do not disturb sleep (Carskadon & Herz, 2004).

In our studies, we experimentally established an association between the odor and the new memory trace by presenting the odor via a face mask connected to an olfactometer while participants performed our declarative or extinction learning tasks. When participants went to bed for post-learning sleep they wore the mask too. For auditory cueing, sounds were first negatively learned and presented again during sleep by loudspeakers. As soon as we detected our target sleep stage in the online EEG, we presented the TMR cue. Importantly, these presentations of the TMR cue must immediately be stopped if the participant enters another sleep stage, moves, or wakes up for the proper interpretation of the post-sleep behavioral results.

As a control condition, sleep periods with the presentation of odorless vehicle (1,2-propanediol) used to ensure that possible effects on memory are caused by the odor and not by sleep alone. In these control groups, the background odor is also presented during learning and the target sleep stage also needs to be identified in the online EEG. The only difference

consists in the presentation of the odorless vehicle instead of the cue odor. For auditory reactivation, a comparison was made between sound on-periods and immediately followed intervals of silence, sound off-periods.

3.3 Polysomnographic recordings and sleep scoring

PSG is an indispensable method in sleep research, since the online display of the participant's sleep informs the researcher about the sleep stage the participant is in and enables them to re-present cues associated with learning during the sleep stage of choice.

Sleep was recorded by standard PSG, including EEG, electrooculogram (EOG), and electromyogram (EMG) electrodes. All derivations were recorded from Ag-AgCl electrodes. EEG electrode sites were selected according to the International 10–20 System. EOG electrodes were placed above and below left eye for vertical EOG, and above the right and below the left eye for horizontal EOG. EMG electrodes were attached to the chin.

PSG data was preprocessed according to the "Manual for the Scoring of Sleep and Associated Events" (Iber et al., 2007): EEG signals were sampled at 200 or 500 Hz, re-referenced to a nose or contralateral mastoid electrodes and filtered between 0.3 and 35 Hz. The channels used for scoring are F3, F4, P3, P4, O3, and O4. In order to determine the exact sleep architecture for each participant and to verify that all TMR trials were presented in the target sleep stage, polysomnographic recordings were always scored offline by at least two experienced researchers in addition to the rough online identification of sleep stages. In case of differences in sleep scorings a third experienced researcher was consulted. The sleep stages scored were: wake after sleep onset (WASO); NREM sleep stages N1, N2, and N3, with sleep stage N3 defining SWS; REM sleep; and movement. These stages were relativized on the total sleep time to take into account different sleep lengths.

4 Manuscripts

The five studies that were conducted to investigate the above mentioned research questions are described in the next five manuscripts forming this chapter. First, the impact of olfactory TMR on declarative memory consolidation and associated changes in sleep EEG parameters were investigated. Since odors were processed actively during sleep, we examine differences in the neural correlates of olfactory processing during wakefulness compared with sleep in the second study. Third, after examining sleep and declarative memory, we investigated the relation between TMR and emotional memory consolidation. In the fourth manuscript, we conducted a study to examine if the enhancing effect of TMR on declarative memory is also applicable to behavioral psychotherapeutic learning. Fifth and last, we further determined the role of sleep in emotional processing by answering the question why dysfunctional emotional states profit from the deprivation of sleep. This study was conducted in a healthy sample as preliminary work to explore if this effect is reflected at all in neural correlates, and needs further application to patients with affective disorders.

4.1 Study 1: Reactivating memories during sleep by odors: Odor-specificity and associated changes in sleep oscillations

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4.1.1 Abstract

Memories are reactivated during sleep. Re-exposure to olfactory cues during sleep triggers this reactivation and improves later recall performance. Here, we tested if the effects of odor-induced memory reactivations are odor-specific, that is, requiring the same odor during learning and subsequent sleep. We also tested whether odor-induced memory reactivation affects oscillatory EEG activity during sleep, as a putative mechanism underlying memory processing during sleep. Participants learned a visuospatial memory task under the presence of an odor. During subsequent SWS, the same odor, a different odor, or an odorless vehicle was presented. We found that odor re-exposure during sleep significantly improves memory only when the same odor was presented again, whereas exposure to a new odor or the odorless vehicle had no effect. The memory-enhancing effect of the congruent odor was accompanied by significant increases in frontal delta (1.5–4.5 Hz) and parietal fast spindle (13.0–15.0 Hz) power as well as by an increased negative-to-positive slope of the frontal slow oscillation. Our results indicate that odor-induced memory reactivations are odor specific and trigger changes in slow-wave and spindle power possibly reflecting a bottom–up influence of hippocampal memory replay on cortical slow oscillations as well as thalamo-cortical sleep spindles.

4.1.2 Introduction

Olfactory stimuli are potent cues for memories. In his oeuvre *En recherche du temps perdu*, Marcel Proust elegantly describes how the smell and taste of a tea-dipped cake instantly reactivate a highly detailed scene of the protagonist's childhood, which he had not recalled for a long time. This efficacy of odors as memory cues has been confirmed in experimental

studies. When stimuli have been learned in the presence of a contextual odor, retrieval of the stimuli was improved when the olfactory stimulus was also present during retrieval testing (Smith, 1992; Schab, 1990, 1991). Importantly, olfactory context effects are odor specific: After learning under the presence of a positive or negative odor, retrieval performance only increased when the same odor was presented again during retrieval testing (Schab, 1990; Cann & Ross, 1989).

In recent studies, we applied the approach of odor cueing to reactivate memories during sleep (Diekelmann, Büchel, Born, & Rasch, 2011; Rasch, Büchel, Gais, & Born, 2007). Sleep promotes memory consolidation, and it is widely assumed that the beneficial effect of sleep on memory relies on memory reactivations during SWS (Oudiette & Paller, 2013; Rasch & Born, 2013; Diekelmann & Born, 2010). According to this concept, hippocampal memory reactivations facilitate the gradual integration of memories from hippocampal into neocortical networks for long-term storage. This process occurs in close coordination with slow oscillatory and fast spindle activity during SWS (Mölle & Born, 2011). Reactivations embedded in spindles during the excitable up state of slow oscillations have been proposed as a mechanism supporting the hippocampo-to-cortical transfer of reactivated memory information (Ngo, Martinetz, Born, & Mölle, 2013; Bergmann, Mölle, Diedrichs, Born, & Siebner, 2012). Indeed, experimentally inducing memory reactivation by re-exposure to a contextual olfactory memory cue during SWS activated hippocampal areas during sleep and resulted in improved memory recall the next day (Diekelmann et al., 2011; Rasch et al., 2007). This concept has received further support by recent findings indicating that reactivating memories during sleep by auditory cueing leads to a strengthening of individual memory traces, suggesting a high degree of specificity of the effects of reactivation on memory consolidation during sleep (Oudiette, Antony, Creery, & Paller, 2013; Antony, Gobel, O'Hare, Reber, & Paller, 2012; Rudoy, Voss, Westerberg, & Paller, 2009). Although a first study on creativity suggests that odor effects on reactivation are absent when different odors are used before and during sleep (Ritter, Strick, Bos, van Baaren, & Dijksterhuis, 2012), the specificity of olfactory cueing for memory consolidation processes during sleep has not yet been examined in previous studies.

Here, we tested the specificity of olfactory cueing during sleep on memory consolidation. Participants learned the position of card pairs in a two-dimensional object-location task under the presence of either a positive or negative odor. During subsequent SWS, the same odor or the other odor was presented. We hypothesized that only re-exposure to the same odor effectively increases sleep-related memory consolidation, resulting in

improved memory performance the next day. In addition, we predicted that these reactivation induced changes in memory are associated with changes in sleep parameters implicated in sleep-dependent memory consolidation processes according to the active system consolidation hypothesis (Rasch & Born, 2013), namely, slow delta, delta, and fast spindle power. For further fine-grained exploratory analysis, we calculated the slope, amplitude, and number of the slow oscillations.

4.1.3 Methods

Participants. Thirty-six nonsmoking healthy participants naive to the experimental protocol participated in the study (12 men, mean age = 23.4, SD = 3.2 years, range = 19–32 years). They were divided into three groups (n = 12 per group) depending on the congruency of the odors presented during learning and during sleep: "congruent group" (same odor during learning and sleep), "incongruent group" (different odors during learning and sleep), and "vehicle group" (odor during learning and odorless vehicle during sleep). Data from three participants (two men, one woman; one in the congruent group and two in the incongruent group) had to be excluded from the EEG analysis because of technical problems with the EEG recordings resulting in 33 participants for EEG data analysis. Age (F(2,33) = 0.08, p > .90) and sex (F(2,33) = 0.35, p > .70) distributions were highly comparable between groups. Participants were in good physical and mental health condition according to a routine examination, did not take any medication at the time of the experiments, and reported a normal sleep–wake cycle with habitual bedtimes starting between 11:00 p.m. and 1:00 a.m. and ending between 6:00 and 8:30 a.m. They had not been on night shift and did not have any major sleep disturbances during 6 weeks before the experiment. Any nasal infections were excluded on the days of the experiments. Participants were habituated to the experimental setting by spending an adaptation night in the sleep laboratory under the conditions of the experiment including the placement of electrodes and of the nasal mask used for delivery of odors during sleep. On experimental days, participants were instructed to get up at 7:00 a.m., not to take any naps, and not to ingest alcohol- or (after 3:00 p.m.) caffeine-containing drinks. Written informed consent was obtained from all participants before participation. The experiment was approved by the ethics committee of the University of Lübeck.

Memory task. The two-dimensional object-location memory task resembles the game "concentration" and consists of 15 card pairs showing colored pictures of different animals

and everyday objects. Performance on this type of task relies on temporal lobe structures including the hippocampus (Sommer, Rose, Gläscher, Wolbers, & Büchel, 2005; Kessels, de Haan, Kappelle, & Postma, 2001). Throughout the task, all possible spatial locations are shown as gray squares on a 15-in. flat screen ("the back of the cards"). The locations are geometrically ordered in a checkerboard-like fashion.

At learning, the first card of each card pair was presented alone for 1 sec followed by the presentation of both cards for 3 sec. After an ISI of 3 sec, the next card pair was presented in the same way. The whole set of card pairs was presented twice. Immediately after these two runs, recall of the spatial locations was tested using a cued recall procedure, that is, the first card of each pair was presented and the participant had to indicate the location of the second card with a computer mouse. Visual feedback was given in each case by presenting the second card at the correct location for 2 sec independent of whether the response was correct or not, to enable reencoding of the correct location of the card pair. The cued recall procedure was repeated until the participant reached a criterion of 60% correct responses. After presenting a card pair, both cards were replaced by gray squares again, so that guessing probability remained the same throughout each run. The odor was delivered in a stimulus-locked way, starting with the onset of the presentation of the first card of each pair and stopping when presentation of both cards ended.

At retrieval testing the next morning, the same cued recall procedure was used during the learning phase, but without odor presentation. To indicate overnight memory consolidation, we used the percentage of correctly recalled card locations at retrieval, with performance on the last run during learning set to the baseline value of 100%. Note that this measure yields values of >100% if more card locations are recalled at retrieval testing than during learning. (Values of >100%, however, do not reflect "true gains" in memory because feedback was given during the last learning trial.) In Table 1, overnight changes are additionally indicated as absolute difference between the number of recalled card locations at retrieval minus performance at learning.

Odor delivery and substance. We used two highly distinct olfactory stimuli in the experiment: Odor A was isobutyraldehyde (IBA, unpleasant; Sigma-Aldrich, Munich, Germany; similarly used in Diekelmann et al., 2011), and Odor B was citral (pleasant; Sigma-Aldrich; tested in pilot studies). Both odors were diluted in odorless mineral oil (1, 2-propanediol; Sigma-Aldrich) at a concentration of 1:100 (citral) and 1:200 (IBA). The odorless mineral oil served as stimulus in the control condition. The experimental odors were delivered via a 12-channel

computer-controlled olfactometer designed after Lorig (2000). Room air was filtered before entering the system, and airflow was held constant at 3 L/min. To avoid tactile or thermal shifts associated with odor onset, half of the air stream was presented continuously to the participant, and only the other half was switched between room air and vehicle or odor presentation by computer-controlled valves. The olfactometer was placed in a separate room (adjacent to the participant's room) and was connected to the participant's mask via teflon tubes, which allowed regulating the odor stimulation without disturbing the participant. The participant received the odor via a small nasal mask, which assured constant stimulation but permitted normal breathing. A 1-mtube with a 12.6-ml volume connected the glass bottles containing the stimulus fluids with the mask, thus allowing rapid odor onset and offset times of 300–500 msec.

Table 1. Performance on the two-dimensional object-location task.

	Congruent odor	**Incongruent odor**	**Vehicle**	***F*(2,33)**	***p***
Number of trials before sleep	3.1 ± *0.4*	3.5 ± *0.5*	2.3 ± *0.5*	1.8	0.19
Recalled card pairs before sleep	9.5 ± *0.3*	10.3 ± *0.3*	10.0 ± *0.3*	2.0	0.16
Change in recalled card pairs (before/after sleep)	+ 0.9 ± *0.6*	- 0.6 ± *0.4*	- 0.7 ± *0.3*	4.5	0.02*

The task included 15 card pair locations. Learning trials were repeated until participants reached a learning criterion of 60% correct responses. Number of trials to reach the criterion and number of card locations recalled at learning are indicated. Change denotes the difference between retrieval performance after sleep and performance at the criterion trial at learning. Data are means ± SEM. Right columns indicate *F* and *p* values for one-way ANOVA.
*$p < .05$.

Design and procedure. Half of the participants learned the two-dimensional object-location task under the presence of Odor A. The other half of the participants received Odor B during learning. In a balanced between-participant design, either the same odor ("congruent group") or the other odor ("incongruent group") was delivered during subsequent SWS. A third group received the odorless mineral oil ("vehicle group") during SWS. No odor was presented during retrieval testing the next morning (see Figure 1A).

Sessions started at 8:30 p.m. with the application of electrodes for standard polysomnography and of the nasal mask. Next, participants performed an odor detection test with the odor applied during learning, to ensure normal olfactory sensitivity. The learning phase of the visuospatial two-dimensional object-location memory task started at 9:30 p.m. The experimental odor was presented time-locked to the presentation of the stimuli to be learned via a nasal mask. The odor detection test was repeated after the learning phase. At 11:00 p.m., participants went to bed and were allowed to sleep for 7.5 hr. The olfactory stimuli were presented during SWS in the first 3 hr after sleep onset. Presentations started as soon as online polysomnographic recordings indicated more than 20% delta waves (i.e., the presence of SWS) during a 30-sec period. The stimulation was interrupted whenever polysomnographic signs of arousal, awakening, or changes in sleep stage appeared. The experimenter was entirely unaware whether odor or vehicle was applied on a given night. In each experimental session, the olfactometer contained Odor A, Odor B, and the vehicle, and the selection was performed automatically by a preprogramed algorithm unknown to the experimenter. Stimulation followed an alternating pattern of 30-sec on-phases/30-sec off-phases to reduce habituation. Participants were awakened at approximately 6:30 a.m. from nonrapid eye movement (NREM) sleep stage 2 or 1, and the nasal mask and electrodes were removed. If these sleep stages were not present at 6:30 a.m., we waited until the next appearance of sleep stage 2 or 1 to awaken participants. About 30 min later, recall was tested on the memory task, without any odor presentation.

As a control measure of vigilance, the participants' RTs were measured before learning and before retrieval. RTs were assessed by a standardized test that required pressing a button as fast as possible whenever a large red disk appeared on a computer screen (as described in Little, Johnson, Minichiello, Weingartner, & Sunderland, 1998). In 40 trials, the participants fixated their gaze on a cross, displayed for 50–1000 msec on a white screen. Then, in 35 trials, a red disk appeared, and in five random no-go trials, the screen remained white.

Sleep and EEG recordings. Sleep was recorded by standard polysomnography (Rechtschaffen & Kales, 1968). EEG was recorded from six scalp (Ag–AgCl) electrodes (F3, F4, C3, C4, P3, and P4; according to the International 10–20 System) and a nose reference. EEG signals were filtered between 0.15 and 35.0 Hz and sampled at 200 Hz. Additionally to the online identification of sleep stages, polysomnographic recordings were scored offline by two experienced technicians. The sleep stages scored were wake after sleep onset (WASO);

NREM sleep stages 1, 2, 3, and 4, with sleep stages 3 and 4 defining SWS; REM sleep; and movement.

For a more fine-grained exploratory analysis of immediate effects of odor cueing during SWS, EEG recordings were subjected to power spectral analysis. Data of the 30-sec on-and-off phases of odor and vehicle stimulation were separated each into three blocks of artifact-free EEG including 2,048 data points each (≈10.2 sec) with an overlap of 205 data points between blocks. A Hanning window was applied on each 2,048-point block before calculating power spectra using fast Fourier transformation (FFT) with a resolution of 0.2 Hz. Individual mean power in the following EEG bands was determined for the odor-on and odor-off periods: frontal slow delta (0.5–1.5 Hz), frontal delta (1.5–4.5 Hz), frontal slow spindle (11.0–13.0 Hz), and parietal fast spindle (13.0–15.0 Hz) bands.

Because recordings from each pair of electrodes (F3 and F4; C3 and C4; P3 and P4) revealed the same results, data were collapsed across both hemispheres. The blocks of odor-on and odor-off periods were used to calculate the percent change of spectral power such that power during the first 10-sec interval of the odor-on period was expressed as percentage of the power during the last 10-sec interval of the preceding odor-off period (set to 100%).

Identification of slow oscillations and slope analysis. In addition to spectral EEG power, we identified discrete slow oscillations and calculated their slopes during odor-on and odor-off intervals. The slopes of the slow oscillation are considered a sensitive measure of synchronization of cortical activity and are possibly related to network synaptic connectivity and its changes over time (Vyazovskiy, Cirelli, & Tononi, 2011). Slow oscillation detection and slope calculation were performed in frontal recording sites, as described previously (Bölsterli et al., 2011; Riedner et al., 2007). In brief, artifact-free EEG data were low-pass filtered at 30.0 Hz and band-pass filtered between 0.5 and 4.0 Hz (stopband of 0.1 and 10.0 Hz) using a Chebyshev Type II filter (MATLAB, The Math Works, Inc., Natick, MA). The chosen filter parameters provided minimal amplitude and wave shape distortion. For each frontal channel (F3, F4), individual negative half-waves were identified. A half-wave was defined as the negative deflection of the EEG between two consecutive zero crossings. We considered only those half-waves whose consecutive zero crossings were separated by 0.25–1.0 sec (i.e., a frequency between 0.5 and 2.0 Hz), which had a corresponding quarter-wave from the negative peak to the next zero crossing lasting more than 0.11 sec (<2.25 Hz) and a minimal amplitude of −75 μV. For these individually identified slow oscillations, we calculated slopes from the negative peak to the next zero crossing (negative-to-positive

slopes) and slopes from the previous zero crossing to the negative peak (positive-to-negative slopes). Whereas positive-to-negative transitions of the surface EEG have been associated with the onset and synchronicity of neuronal down states, negative-to-positive transitions might be more related to the onset of the subsequent up state (Vyazovskiy et al., 2009). As in the power analysis, the relative change in numbers, slopes, and amplitudes in slow oscillations was calculated for the first 10-sec interval of the odor-on period with reference to the last 10-sec interval of the preceding odor-off period (set to 100%).

Spindle analysis. Spindle counts and density during odor-on and odor-off intervals were analyzed because of their well-known relationship with overnight retention of memories (Saletin, Goldstein, & Walker, 2011; Fogel, Nader, Cote, & Smith, 2007; Nishida & Walker, 2007; Gais, Mölle, Helms, & Born, 2002). Discrete spindles are a characteristic feature of sleep stage 2 and occur also in SWS but are virtually absent during REM sleep. Slow (11.0–13.0 Hz) and fast (13.0–15.0 Hz) spindles were separately identified at the six EEG recording sites based on an algorithm adopted from previous studies (Gais et al., 2002; Schimicek, Zeitlhofer, Anderer, & Saletu, 1994). In brief, power was extracted in the frequency bands of interest (11.0–13.0 Hz; 13.0–15.0 Hz), and the events were counted as spindles for which the power signal exceeded a fixed threshold (±10 μV) for an interval lasting 0.5–3 sec. Spindles were counted separately in each channel during 30-sec NREM EEG segments free of movement artifacts (maximal difference in EMG activity of <150 μV). Mean spindle counts were calculated by averaging spindle counts of all six channels. To calculate mean spindle density, mean spindle counts were divided by the number of analyzed NREM 30-sec epochs. One participant of the odorless vehicle group had to be excluded because the algorithm did not detect any discrete sleep spindles during the odor-on and odor-off periods. Thus, 32 participants were included in the spindle analysis. The two separate spindle bands were chosen based on previous studies, which demonstrated the presence of two kinds of spindles in humans possibly linked to different aspects of cognitive function, that is, slow spindles that prevail over frontal cortex and show greater topographical variability than the fast spindles that concentrate over parietal cortex (Mölle, Bergmann, Marshall, & Born, 2011; Schabus et al., 2007; Zeitlhofer et al., 1997).

Arousal analyses. We performed analyses on EMG power, EMG arousal counts, and EEG arousal counts to control for arousal-induced changes in sleep parameters. Three additional participants had to be excluded from the EMG analyses because of loss of the EMG signal at

the beginning of the night, resulting in 30 participants (congruent: n = 10, incongruent: n = 9, vehicle: n = 11).

EMG arousal counts were analyzed using the EMG channel. Data were rectified, a moving average of 125 msec was applied, and a baseline of 500 msec before each segment was subtracted. Signal peaks above 40 μV were counted as EMG arousal. The number of EMG arousals during the 10-sec odor-off periods was subtracted from the number of EMG arousals during the 10-sec odor-on periods and relativized on the overall number of stimulations. We used the same method over the total sleep period to detect the number of EMG arousals for the entire night and relativized the number of EMG arousals on the total sleep time.

For the power analysis of the EMG signal, we applied a notch filter (50 Hz) and calculated the FFT in the 10- to 100-Hz range (Fridlund & Cacioppo, 1986). We set the upper restriction to 100 Hz because of our sampling rate of 200 Hz. We performed this FFT similar to the FFT for EEG analysis and calculated the percent change in power during the first 10 sec of the odor-on interval with power in the preceding last 10 sec of the odor-off interval set to 100%.

In addition to the EMG analysis, we also counted EEG arousals during 10-sec and 30-sec odor-on and odor-off intervals by visual inspection of the six EEG channels. We used the EEG arousal scoring rules of the American Sleep Disorders Association and Sleep Disorders Society (ASDA, 1992). An EEG arousal was counted if there was a change to alpha, theta, or frequencies greater than 16 Hz, which lasted for at least 1.5 sec (De Gennaro, Ferrara, & Bertini, 2001). EMG was not considered because we only analyzed NREM sleep. To demonstrate that odors do not cause differences in arousals of higher frequencies (above 16 Hz), we additionally applied a high-pass filter with 16 Hz to our data and counted the remaining arousals (an arousal was counted if it lasted longer than 1.5 sec). We calculated the absolute difference in the number of both types of EEG arousals between odor-on and odor-off periods (rather than percentages to avoid division by zero, as several participants had no EEG arousal at all during these periods).

Data analyses. Data were analyzed using 2 × 3 ANOVA with the factors "Odor during learning" (Odor A vs. Odor B) and "Group" (congruent odor vs. incongruent odor vs. vehicle). For significant main effects or interactions, post hoc pairwise comparisons were performed using the least significant difference method. If Mauchly sphericity test reached significance, we displayed degrees of freedom and *p* values that were Greenhouse–Geisser

corrected. Correlation analyses were conducted using Pearson correlation. A p value < .05 was considered significant.

4.1.4 Results

Memory performance. As expected, administration of the same odor was critical for the memory-improving effect of odor re-exposure during sleep. When the same odor was presented during learning and subsequent SWS (congruent group), participants recalled 110.2 ± 5.8% of the card pairs they had learned before sleep. Participants who received a different odor during learning than during sleep (incongruent group) recalled only 94.5 ± 3.5% of the card pairs, which was comparable with those who received the odorless vehicle during SWS (vehicle group, 93.4 ± 3.3%). Recall performance between the three groups differed significantly (main effect Group: $F(2,33) = 4.7$, $p = .02$; results for absolute differences are indicated in Table 1). Post hoc pairwise comparisons revealed that performance in the congruent group was significantly higher as compared with both the incongruent ($p = .02$) and vehicle ($p = .01$) groups, whereas the incongruent and vehicle groups did not differ ($p > .80$; see Figure 1B). The results were not affected by the valence of the odor presented during learning ($p > .80$ for main and interaction effects of Odors A and B). Memory performance in the incongruent and vehicle groups was roughly comparable with performance after a night of sleep without any intervention as reported previously (90.9 ± 4.5%; see Rasch et al., 2007, supporting material, p. 6).

The three experimental groups did not differ in their learning performance before sleep, neither in the number of card pairs recalled in the last learning trial nor in the number of trials to achieve the learning criterion of 60% (both ps ≥ .16, Table 1). The number of trials to reach the learning criterion was not correlated with changes in memory improvement ($r = .26$, $p > .15$), frontal delta power ($r = -0.26$, $p > .15$), frontal slow delta power ($r = -0.16$, $p > .30$), parietal fast spindle power ($r = .11$, $p > .50$), and slow oscillation slopes ($r = -0.07$, $p > .60$).

Furthermore, participants were asked after the experiment whether they had received the same odor during sleep as during learning, a different odor, or no odor. Only 1 of 36 participants gave the correct answer, 16 gave incorrect answers, and 15 participants indicated "I don't know."

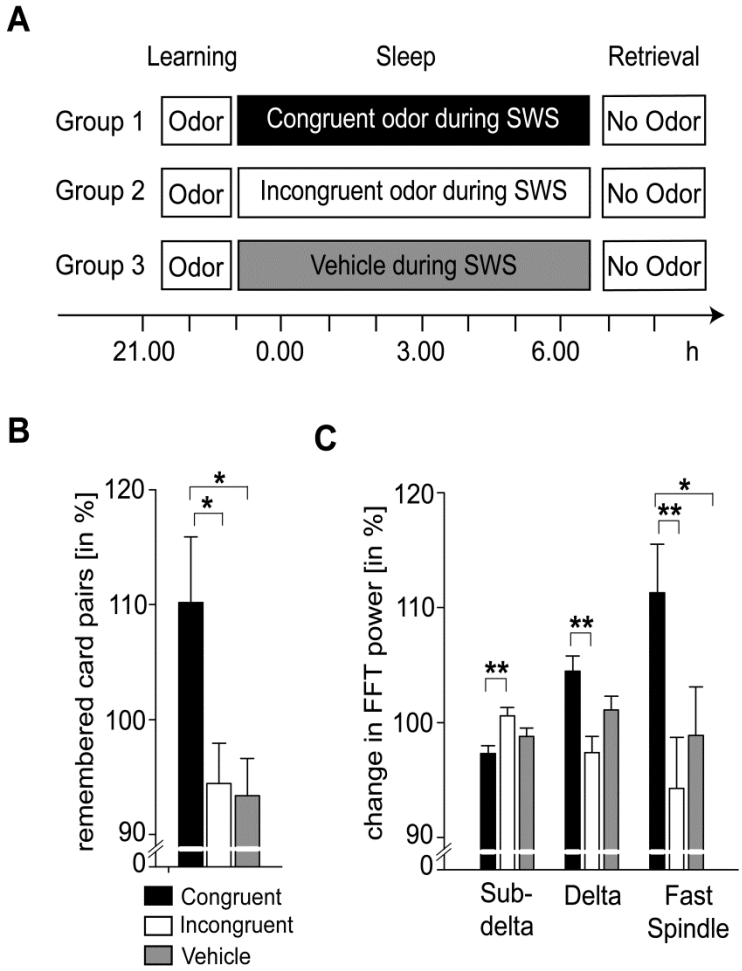

Figure 1. (A) Procedure. Participants learned the two-dimensional object-location task under the presence of a specific odor. According to the group, the same odor, a novel odor, or an odorless vehicle was presented during the first two periods of subsequent SWS. Retrieval took place the morning after sleep without odor. (B) Percentage of remembered card pairs after sleep relative to the number of correctly identified card pairs during learning before sleep. Values greater than 100% indicate more remembered card pairs during retrieval than during learning. (C) Changes in relative EEG power during the first 10 sec of odor-on intervals compared with the last 10 sec of odor-off intervals. Data for slow delta (0.5–1.5 Hz) and delta (1.5–4.5 Hz) power are retrieved from frontal electrodes. Data for fast spindle power (13.0–15.0 Hz) are retrieved from parietal electrodes. For B and C, displayed values are mean values ± SEM. *p* values from planned pairwise post hoc comparisons are indicated (**p* < .05, ***p* < .01).

Sleep architecture. The three groups did not differ in total sleep time ($p > .70$), and there were also no differences in the percentage of time they spent in sleep stages S1, S2, SWS, REM sleep, or awake (all *p*s ≥ .17, Table 2). The number of movements during the whole night revealed a trend for significance between groups ($F(2, 30) = 3.27$, $p = .052$), with highest values in the incongruent odor group (1.4 ± 0.35%; congruent group: 0.77 ± 0.14%; vehicle group: 0.62 ± 0.12%). Post hoc pairwise comparisons revealed a significant difference between the incongruent and vehicle conditions ($p = .02$) and marginal significance between the congruent and incongruent conditions ($p = .06$), whereas the congruent and vehicle

conditions did not differ ($p > .60$; see Table 2). When including the number of arousals as covariate for the difference of memory improvement between groups, the difference between groups was still significant ($F(2, 29) = 6.51$, $p = .005$) with a similar pattern of performance (congruent: 112.76 ± 4.45%, incongruent: 94.47 ± 4.72%, vehicle: 92.03 ± 4.55%).

Table 2. Sleep parameters.

	Congruent odor	**Incongruent odor**	**Vehicle**	***F* *(2,33)***	***P***
WASO %	1.2 ± *0.5*	6.2 ± *3.0*	3.5 ± *0.9*	1.8	0.17
S1 %	5.0 ± *0.6*	5.4 ± *1.0*	7.0 ± *0.6*	1.9	0.17
S2 %	55.9 ± *2.4*	53.1 ± *2.6*	51.9 ± *1.8*	0.8	> 0.40
SWS %	16.2 ± *2.0*	17.4 ± *1.6*	17.2 ± *1.8*	0.1	> 0.80
REM %	20.1 ± *1.6*	17.3 ± *1.8*	19.5 ± *1.5*	1.3	> 0.20
Movement %	0.8 ± *0.14*	1.4 ± *0.35*	0.6 ± *0.12*	3.27	0.052
Sleep time [min]	445 ± *8.2*	447 ± *13.8*	456 ± *13.6*	0.3	> 0.70
SOL [min]	25.0 ± *2.4*	35.1 ± *9.2*	31.0 ± *4.6*	0.7	> 0.40
Sleep efficiency%	93.6 ± *0.9*	86.8 ± *3.5*	90.2 ± *1.4*	2.3	0.12
Number of stimulations	65.8 ± *6.0*	56.7 ± *4.9*	59.4 ± *5.4*	0.7	> 0.40

WASO, S1, S2 (NREM sleep stages 1 and 2), SWS (combined sleep stages 3 and 4), and REM sleep in percent of total sleep time (sleep time). Number of arousals in percent of the total time scored as sleep. SOL = sleep onset latency. Data are means ± SEM. Right columns indicate *F* and *p* values for one-way ANOVA.

Effects of odor-induced reactivation on oscillatory activity during sleep. In accordance with our hypothesis, exposure to the same odor during learning and subsequent SWS increased oscillatory activity in the delta and fast spindle bands shortly after odor onset, whereas slow delta power was surprisingly reduced (see Table 3 and Figure 1C; for all other frequency bands at all electrode sites, see Supplementary Tables 1–4).

During the first 10-sec interval of the odor-on period, power in the 1.5- to 4.5-Hz delta band over frontal electrodes significantly increased to 104.5 ± 1.2% in the congruent group, with the preceding 10 sec of the odor-off interval set to 100% ($t(10) = 3.68$, $p = .004$, one-

sample t test). In contrast, no changes in delta power were observed during administration of an odor different from that during learning (incongruent group, 97.4 ± 1.6%; $t(9) = -1.61$, $p = .14$) or an odorless vehicle (vehicle group, 101.1 ± 1.1%; $t(11) = 1.0$, $p > .30$) during sleep. The change in delta power differed significantly between the three experimental groups (ANOVA: $F(2, 30) = 7.13$, $p = .003$). Post hoc group-wise comparison revealed a stronger increase in delta activity in participants of the congruent group compared with the incongruent group ($p = .001$) and a trend for a stronger increase compared with the vehicle group ($p = .07$). The difference between the incongruent and vehicle groups also almost reached significance ($p = .054$; Figure 1C). An additional analysis of the delta band using the 1.0- to 4.5-Hz range revealed similar results (overall: $F(2, 30) = 3.43$, $p = .046$). The reported differences in changes in delta power between the groups were not related to differences in baseline power: Values of the last 10 sec of the odor-off interval did not differ between the three groups (all ps > .50 for all frequency bands of interest). In addition, including these odor-off values as a covariate did not alter any of the reported results.

The increase in delta activity during odor-on periods in the congruent group was accompanied by a parallel reduction of 0.5- to 1.5 Hz-slow delta power over frontal electrodes in the congruent group for odor-on intervals (97.3 ± 0.7%, $t(10) = -4.07$, $p = .002$, one-sample t test). Similarly, the vehicle group showed a decrease in slow delta power (98.7 ± 0.6%, $t(11) = -2.17$, $p = .052$), whereas no changes in slow delta power were observed during administration of a novel odor (100.6 ± 0.8%, $t(9) = 0.71$, $p > .40$). The overall difference in slow delta power between the three conditions was also significant (ANOVA: $F(2, 30) = 5.50$, $p = .009$). Post hoc tests indicated a significant difference between the congruent and incongruent groups ($p = .009$). The congruent and vehicle groups did not differ ($p = .13$), whereas marginal significance resulted for the difference between the vehicle and incongruent groups ($p = .07$; Figure 1C). An additional analysis of the slow delta band using the 0.5- to 1.0-Hz range revealed similar results (overall: $F(2, 30) = 7.26$, $p = .003$).

In addition, we observed a significant increase in 13.0- to 15.0-Hz fast spindle power over parietal electrodes during odor-on periods in the congruent group (111.2 ± 3.8%; $t(10) = 2.96$, $p = .014$, one-sample t test), whereas no changes occurred in the two control groups (incongruent group: 94.2 ± 4.1%, $t(9) = -1.39$, $p = .20$; vehicle group: 98.9 ± 4.6%, $t(11) = -0.24$, $p > .80$). The overall difference between the three experimental conditions was significant (ANOVA: $F(2, 30) = 4.21$, $p = .03$). Post hoc pairwise comparisons revealed significant differences between the congruent compared with the incongruent ($p = .009$) and the vehicle ($p = .043$) groups, whereas the incongruent and vehicle groups did not differ ($p >$

.40; Figure 1C). Using a spindle algorithm to detect spindle density averaged over all channels, no significant group differences were found for fast spindle counts ($F(2, 29) = 0.78$, $p > .40$) or density ($F(2, 29) = 0.13$, $p > .80$; for fast spindle density values of the single channels, see Supplementary Table 5).

In contrast to parietal fast spindle power, changes in frontal slow spindle power over frontal electrodes did not differ between the three groups ($F(2, 30) = 0.21$, $p > .90$). Furthermore, there were no significant correlations between memory performance and changes in delta ($r = .28$, $p = .12$), slow delta ($r = -0.25$, $p = .17$), or fast spindle ($r = .04$, $p > .80$) power.

Table 3. Percentage of changes in FFT power.

	Congruent odor	**Incongruent odor**	**Vehicle**	***F* (2,30)**	***P***
Slow Delta	97.3 ± *0.7*	100.6 ± *0.7*	98.8 ± *0.7*	5.5	0.009**
Delta	104.5 ± *1.3*	97.4 ± *1.4*	101.1 ± *1.2*	7.1	0.003**
Fast Spindle	111.3 ± *4.2*	94.3 ± *4.4*	98.9 ± *4.0*	4.2	0.03*

Data are retrieved from frontal (slow delta and delta band) and parietal (fast spindle band) electrodes during the first 10 sec of the odor-on interval compared with the last 10 sec of the preceding odor-off interval set to 100%. Data are means ± SEM. Right columns indicate *F* and *p* values for one-way ANOVA. *$p < .05$; **$p < .01$.

The relative increase in delta power together with a relative reduction in slow delta power during odor-on periods in the congruent group might reflect a shift in the number and/or morphology of slow oscillations. To test this, we detected and analyzed individual slow oscillations (>75 μV) in frontal electrodes during the odor-on and odor-off intervals. For negative-to-positive slopes, relative changes in slopes of the frontal EEG slow oscillations were significantly higher in the congruent group (102.8 ± 2.1%) as compared with the incongruent (95.2 ± 3.5%) and the vehicle (90.4 ± 3.7%; $F(2, 30) = 4.03$, $p = .03$) groups. Post hoc tests indicated only a significant difference between the congruent and vehicle groups ($p = .008$), whereas there was no difference between the congruent and incongruent groups ($p = .11$) or between the incongruent and vehicle groups ($p > .30$; Figure 2A). Neither relative changes in amplitude (99.8 ± 1.9% vs. 98.5 ± 1.7% vs. 98.8 ± 1.4%, respectively; $F(2, 30) = 0.15$, $p > .80$; Figure 2A) nor numbers (107.5 ± 7.2% vs. 96.7 ± 4.3% vs. 96.1 ±

3.2%; $F(2, 30) = 1.57$, $p > .20$) of slow oscillations significantly differed between groups. Also, no differences in slope changes between the three experimental groups were observed for positive-to-negative slopes (99.5 ± 3.5% vs. 93.2 ± 3.4% vs. 97.1 ± 4.9%; $p > .50$). Altogether, this pattern suggests that the shift in power from the slow delta to the delta band primarily originated from an increase in the negative-to-positive slope of the slow oscillations. Furthermore, changes in negative-to-positive slopes of slow oscillations over frontal cortex correlated significantly with improved memory consolidation across sleep ($r = .40$, $p = .02$). Remarkably, this correlation was significant only in the congruent group ($r = .62$, $p = .04$), but not in the incongruent ($r = .28$, $p > .40$) or vehicle ($r = -.04$, $p > .80$; Figure 2B–D) group.

When considering the whole 30-sec period of odor stimulation relative to the preceding 30-sec off period, the group difference remained significant for frontal delta power ($F(2, 30) = 4.57$, $p = .02$), with a significantly higher value for the congruent compared with the incongruent condition ($p = .02$) and also for the vehicle compared with the congruent condition ($p = .01$). Incongruent and vehicle conditions did not differ ($p > .80$). Parietal fast spindle power also differed significantly between conditions ($F(2, 30) = 3.74$, $p = .04$). Post hoc pairwise comparisons revealed only a significant difference between the congruent and incongruent conditions ($p = .01$), whereas the incongruent and vehicle conditions ($p > .30$) as well as the congruent and vehicle conditions did not differ significantly ($p = .09$). Frontal slow delta power revealed no difference between groups ($F(2, 30) = 1.40$, $p > .20$; for detailed values, see Supplementary Table 6). Concerning the changes in frontal slopes, the difference for the entire stimulation interval revealed only a trend for significance ($F(2, 30) = 2.60$, $p = .09$).

Arousal. To exclude that the reported EEG changes during the presentation of the congruent odor reflect increased intrasleep wakefulness instead of increased neural synchrony, we additionally analyzed EMG and EEG arousal responses during the 10-sec odor-on periods with reference to the previous 10-sec odor-off periods. These analyses revealed that arousal responses did not differ between the three experimental conditions, neither in the number of EMG arousals (congruent: −0.43 ± 0.61, incongruent: −0.57 ± 0.42, vehicle: −0.54 ± 0.62; $F(2, 29) = 0.02$, $p > .90$) nor in changes in EMG power (congruent: 97.49 ± 2.44%, incongruent: 102.10 ± 4.38%, vehicle: 96.14 ± 2.01%; $F(2, 29) = 1.07$, $p > .30$).

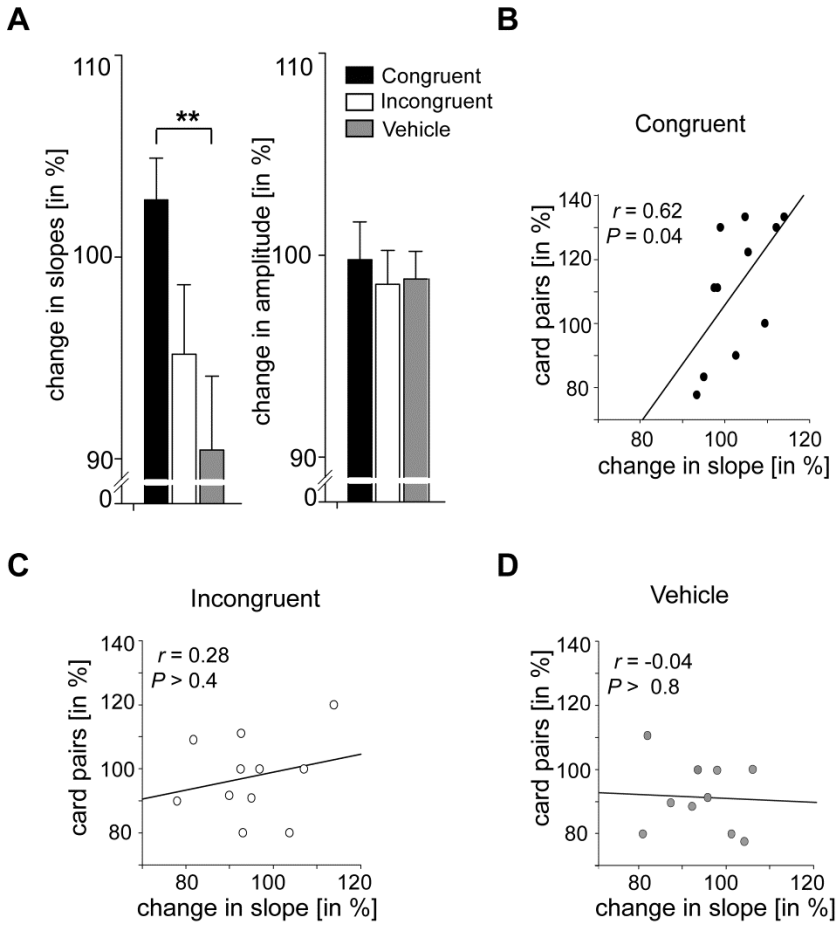

Figure 2. (A) Changes of slopes and amplitudes of slow oscillations between groups in the frontal electrodes during the first 10 sec of odor-on intervals compared with the last 10 sec of odor-off intervals. Displayed values are retrieved from one-way ANOVA, and *p* values from planned pairwise post hoc comparisons are indicated (**$p < .01$). Differences between groups were significant only for slopes ($p = .03$). (B–D) Correlations between memory performance during retrieval (% retrieval) and relative changes in slopes in frontal electrodes for the congruent group (B), the incongruent group (C), and the vehicle group (D). Overnight memory consolidation is indicated as percentage of correctly recalled card locations at retrieval, with performance on the last run during learning set to 100%. Note that this measure yields values of >100% if more card locations are recalled at retrieval testing than during learning.

We could also not find any differences in EEG arousals when considering all arousals between groups for the first 10 sec of the odor-on period compared with the last 10 sec of the odor-off period (congruent: −0.36 ± 0.60, incongruent: −0.20 ± 0.36, vehicle: 0.17 ± 0.34; $F(2, 30) = 0.4$, $p > .60$) or for the full 30-sec odor-on interval compared with the preceding full 30-sec odor-off interval (congruent: −0.09 ± 0.68, incongruent: −0.50 ± 0.72, vehicle: −0.50 ± 0.65; $F(2, 30) = 0.1$, $p > .80$; for mean EEG arousal values, see Supplementary Table 7). Similarly, after high-pass filtering the EEG at 16 Hz and considering only frequencies above 16 Hz, arousal counts for the first 10 sec (congruent: −0.36 ± 0.47, incongruent: −0.50 ± 0.40, vehicle: −0.75 ± 0.30; $F(2, 30) = 0.3$, $p > .70$) and for the full 30-sec interval did not differ significantly between groups (congruent: −0.36 ± 0.61, incongruent: −1.40 ± 0.76,

vehicle: −1.58 ± 0.51; $F(2, 30) = 1.1$, $p > .30$; for mean EEG arousal values greater than 16 Hz, see Supplementary Table 8; for examples of raw EEG data, see Supplementary Figure 1).

Odor stimulation, odor sensitivity, and vigilance. Participants in all conditions underwent on average 60.6 ± 3.1 odor stimulations during the experimental night. The number of stimulations did not differ between groups ($F(2, 33) = 0.73$, $p > .40$). In all three conditions, the number of stimulations was not correlated with changes in retrieval performance (all *p*s > .70).

The odor detection test performed before the experiment proper required participants to indicate the presence or absence of the experimental odor stimulus on 10 trials. The number of correct responses was, on average, 91.1 ± 1.7% and did not differ between odor and vehicle c conditions ($F(2, 33) = 1.10$, $p > .30$). Participants rated on 10-point scales the familiarity, arousal, intensity, valence, and penetrance of the odors. One participant was excluded from this analysis because of data loss. Judgments did not differ between the three experimental groups (all *F*s(2, 32) < 1.81, all *p*s ≥ .18). When divided into groups according to the odor received during learning, appraisals between groups differed. Participants rated the odor citral as more familiar ($t(33) = −4.34$, $p < .001$), more positively valenced ($t(33) = −2.14$, $p = .04$), less arousing ($t(33) = −2.45$, p=.02), and more intense ($t(33)$=−2.27, $p = .03$) compared with the odor IBA. The odors did not differ concerning penetrance ($t(33) = 1.02$, $p > .30$).

Valence ratings of the odors did not correlate with memory performance or relative changes of frontal delta power, frontal slow delta power, parietal fast spindle power, or slow oscillation slopes (all *p*s > .3).

RT on the vigilance task during learning was, on average, 277.9 ± 5.10 msec and did not differ between groups ($F(2, 33) = 2.31$, $p = .12$). RT during retrieval was, on average, 270.22 ± 5.55 msec and also did not differ ($F(2, 33) = 1.40$, $p > .20$).

4.1.5 Discussion

Our results show that re-exposure during SWS to the same odor presented during learning enhances memory performance and triggers an increase in EEG delta and fast spindle power, in comparison with two control groups receiving either another odor or an odorless vehicle during SWS. Our findings indicate that (a) the same odor during learning and sleep is required for reactivating memories during SWS and for improving retrieval of these memories the next

day and that (b) successful reactivation of odor-associated memories during SWS is associated with a specific response of the slow oscillation and fast spindles.

Olfactory stimuli are powerful cues for memories (Willander & Larsson, 2006; Chu & Downes, 2000, 2002; Herz & Schooler, 2002; Herz & Engen, 1996; Herz & Cupchik, 1995; Laird, 1934). The great efficacy of odors to reactivate memories might be a consequence of the close connections of the olfactory cortex to memory-related brain regions like the hippocampus and amygdala. Importantly, there are direct projections from the olfactory cortex to the hippocampus that bypass the thalamus (Gottfried, 2010; Zelano & Sobel, 2005) and might be particularly involved in mediating olfactory stimulation during sleep. Because of these connections, thalamic gating during sleep is expected to affect olfactory processing during sleep to a lesser extent than other sensory modalities. This could result in an increased effectiveness of olfactory cueing during sleep in comparison with memory reactivation induced by other sensory stimuli, for example, auditory cues. Additionally, olfactory stimuli do not disturb ongoing sleep when presented during deeper sleep stages (Carskadon & Herz, 2004).

The memory-improving effect of odor re-exposure during SWS is quite robust and has now been replicated in three independent studies including the current one (Diekelmann et al., 2011; Rasch et al., 2007). These studies have also specified that odor re-exposure during sleep activates the left hippocampus (Rasch et al., 2007) and results in an immediate stabilization of memory traces, even in the absence of REM sleep (Diekelmann et al., 2011). Another research group found that odor re-exposure during sleep after a learning phase also improves creativity (Ritter et al., 2012).

EEG slow-wave activity (SWA) including the 0.5- to 1.5-Hz slow delta and the 1.5- to 4.5-Hz delta band is the hallmark of SWS and has been implicated in declarative memory consolidation during sleep (Diekelmann & Born, 2010). Declarative memory consolidation mainly profits from early, SWS-rich sleep (Drosopoulos, Wagner, & Born, 2005; Plihal & Born, 1999; Yaroush, Sullivan, & Ekstrand, 1971), and learning-dependent increases in SWA have been observed after encoding of declarative and procedural memories (Wilhelm et al., 2011; Huber et al., 2006). Most of the theoretical work on declarative memory consolidation during sleep assumed a crucial role of slow oscillations in this process. Slow oscillations show a prominent spectral peak in the <1-Hz frequency range, whereas the power spectrum particularly of the falling and rising flanks of the slow oscillations includes also faster frequencies in the delta range (>1 Hz). It is proposed that the slow oscillation up state synchronizes hippocampal sharp-wave ripples (which are associated with memory

reactivations) with thalamic spindle activity to optimize strengthening of memories on the cortical level (Mölle & Born, 2011; Diekelmann & Born, 2010). This was confirmed by studies showing that experimentally increasing slow waves by electrical stimulation or tones improves declarative memory consolidation (Ngo et al., 2013; Marshall, Helgadóttir, Mölle, & Born, 2006). Here, we found an increase in delta activity during reactivation of declarative memories, which was accompanied by reduced power in the slow delta band. Further analyses suggested that this shift in power toward higher frequencies primarily reflects an increase in the negative-to-positive slope of slow oscillations largely corresponding to down-to-up-state transition, whereas amplitude of the slow oscillations remained largely unchanged. Thus, this shift in the slow oscillation slopes changes the EEG power from slow delta to delta frequencies while leaving the amplitude and number of the slow oscillations unchanged. Interestingly, the congruent odor changes in delta and spindle power are not accompanied by changes in the number of slow oscillations or discrete sleep spindles. In the case of delta, we believe that the increase in delta activity is induced by a change in slope of the slow oscillations rather than by a change in number or amplitude, which is supported by our slope analysis. For sleep spindles, we can only speculate that the congruent odor increases in spindle power are either only transient or not sufficiently large to induce distinct sleep spindles detected by the spindle detection algorithm. Alternatively, higher spindle power could be indicative of a stronger functional efficacy of sleep spindles, for example, in the temporal grouping of hippocampal sharp-wave ripples, which does not necessarily need to express in a higher number of spindles. The change in slow oscillation slopes significantly predicted memory retrieval after sleep, however, only in the group with congruent odor stimulation, that is, the group with effective reactivation of memories. Slopes of slow oscillations have been associated with processes of synchronization on the neural level in studies using simultaneous recordings of EEG and multiunit activity (Vyazovskiy et al., 2009, 2011). Thus, both the reactivation-induced shift of power toward higher frequencies in the delta band and the associated increase in slow oscillation slopes together with an increase in parietal fast spindle power might reflect an increase in neural synchrony in cortical neurons triggered by induced memory reactivations during sleep. This increased neural synchrony might well favor synaptic plastic processes mediating the observed enhancement in memory. As a speculation, the degree of cortical synchronization induced by reactivation during sleep could be relevant for improvements in offline memory consolidation. Alternatively and/or simultaneously, induced memory reactivation could directly facilitate or trigger other processes supporting the consolidation of the newly learned information during sleep.

Interestingly, the changes that we observed in sleep oscillations on congruent odor reactivation were most pronounced during the first 10 sec of odor stimulation. Similar but weaker changes were visible during the entire 30-sec period of odor presentation, suggesting that external reactivation cues might be most effective in facilitating consolidation processes shortly after stimulation onset, with longer stimulation periods not providing additional benefits.

Whereas it is widely accepted that the neocortical slow oscillation exerts a top–down influence on thalamic and hippocampal activity, which synchronizes thalamo-cortical spindles and hippocampal memory reactivations to the slow oscillation up state (Mölle & Born, 2011; Ji & Wilson, 2007; Wolansky, Clement, Peters, Palczak, & Dickson, 2006), it is currently a matter of debate whether hippocampal memory reactivations and associated sharp-wave ripples can exert a converse "bottom–up" control on spindles and the neocortical slow oscillation. Correlational analyses of the temporal relationships between hippocampal and neocortical activity revealed increases in sharp-wave ripples associated with the developing slow oscillation up state, consistent with the view that sharp-wave ripples contribute to the induction and maintenance of widespread depolarization in cortical networks characterizing the slow oscillation up state (Peyrache, Khamassi, Benchenane, Wiener, & Battaglia, 2009; Mölle, Yeshenko, Marshall, Sara, & Born, 2006; Sirota, Csicsvari, Buhl, & Buzsáki, 2003). However, those analyses basically remain inconclusive with regard to the direction of the influence between neocortex and hippocampus. Assuming that congruent odor presentation during SWS specifically acts to enhance hippocampal memory reactivations and the number of associated sharp-wave ripples (although see Bendor & Wilson, 2012), the present data can be taken as a first hint that hippocampal reactivations by producing an enhanced information transfer to higher cortical networks indeed causally contribute, in a bottom–up manner, to the formation of cortical slow oscillation up states. The effect expressing itself mainly in a steeper slow oscillation slope (and in corresponding increases in delta power) rather than in increased slow oscillation amplitude suggests that hippocampal reactivation primarily contributes to synchronizing activity in distributed neocortical networks during the excitable up state of the slow oscillation. Such influence might help optimizing plastic synaptic processes underlying the storage of reactivated memory information in neocortical regions, although this scenario is in need of further experimental elaboration.

The power changes in the delta band during odor administration are specific to the reactivation of memory-related contents, as no such changes were observed when participants were exposed to a different odor during sleep than during learning. In addition, our study

confirms that unspecific olfactory stimulation during SWS is not sufficient to reactivate memories previously associated with an olfactory context. Only when the same odor was present during learning and subsequent SWS, memory retrieval was improved on the next day. Such context specificity of cueing is known from previous research on olfactory context effects during wakefulness: When participants learned word lists or pictures in a certain odor context, retrieval performance as improved only in the presence of the same odor but not with an odor different from that present during learning (Smith, 1992; Schab, 1990; Cann & Ross, 1989). Concurring with the present findings, the positive or negative valence of the odors was unrelated to the context-related memory improvement in these studies. Furthermore, individual valence ratings of the odors did not correlate with subsequent changes in memory or sleep parameters. Thus, as expected, odor re-exposure during sleep shares properties known from context effects on memory recall during wakefulness, although active retrieval is omitted during sleep.

Although we cannot fully exclude any possible effects of arousals on changes in memory and oscillatory activity, such effects are unlikely given our findings from several additional analyses, namely, visual scoring of EEG arousals, analyses of arousal-related power bands, and analyses of frequencies higher than 16 Hz. Most importantly, the number of arousals was comparable in all experimental groups. Participants who received the same odor during learning and during sleep showed the same number of arousals as participants who received a different odor during sleep than during learning and participants who were presented with an odorless vehicle.

Arousal counts were also comparable when considering only high-frequency arousals with frequencies larger than 16 Hz. Furthermore, groups did not differ in power of any of the frequency bands that are classically associated with arousal responses, namely, in the theta, alpha, beta, and gamma frequency bands. The oscillations for which the congruent odor induced an increase in power, that is, the delta band, fast spindle band, and slow oscillation slopes, are not typically related to arousal responses.

On the contrary, some researchers even define the suppression of delta power and spindle power as a marker of arousals (e.g., Cho, Joo, Koo, & Hong, 2013; Wulbrand, McNamara, & Thach, 1998). Additionally, all of the observed changes in oscillatory activity as well as in memory performance were only evident on cueing with the memory-related congruent odor but not with the unrelated incongruent odor, excluding any unspecific arousal effects on odor stimulation.

Taken together, re-exposure during SWS to an odor that was already present during prior learning improves memory, enhances fast spindle power, and shifts SWA from slower to faster delta oscillations. The latter is reflected in an increase in the slopes of the slow oscillation, which points to increased synchrony at the neuronal level. Thus, we present novel evidence that experimentally induced reactivations of hippocampus-dependent memory shape SWA associated with the consolidation process. The exact mechanisms of this shaping influence need to be elaborated in rodent models of hippocampal memory consolidation.

4.1.6 Acknowledgments

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4.1.8 Supplementing information for: Reactivating memories during sleep by odors: Odor-specificity and associated changes in sleep oscillations

Supplementary table 1. Percentage of changes in all electrodes in FFT power during the first 10 seconds of the odor on-interval with the last 10 seconds of the odor off-interval set to 100%.

	Congruent Odor	**Incongruent Odor**	**Vehicle**	***F*** ***(2,30)***	***p***
Slow Delta	97.7 ± *0.7*	100.7 ± *0.7*	99.4 ± *0.7*	4.5	0.02*
Delta	104.5 ± *1.3*	97.4 ± *1.5*	100.3 ± *1.2*	7.0	0.003**
Theta	103.6 ± *1.4*	99.3 ± *2.7*	100.5 ± *2.5*	0.9	> 0.40
Alpha	101.4 ± *3.0*	100.6 ± *4.5*	103.0 ± *3.5*	0.1	> 0.80
Slow Spindles	103.3 ± *3.3*	100.8 ± *4.7*	101.7 ± *3.3*	0.1	> 0.80
Fast Spindles	111.1 ± *4.3*	95.5 ± *4.3*	100.2 ± *4.3*	3.3	0.05
Beta	98.7 ± *3.2*	99.8 ± *3.3*	100.1 ± *3.0*	0.06	> 0.90
Gamma 1	95.6 ± *4.4*	99.0 ± *3.7*	89.9 ± *6.0*	0.9	> 0.40
Gamma 2	96.0 ± *3.0*	98.3 ± *1.0*	89.0 ± *5.1*	1.8	0.19

Means ± s.e.m. as well as *F* and *p*-values from one-way ANOVAs are indicated. Frequency bands are defined as followed: Slow Delta: 0.5-1.5Hz, Delta: 1.5-4.5Hz, Theta: 4.5-8.0Hz, Alpha: 8.0-11.0Hz, Slow Spindles: 11.0-13.0Hz, Fast Spindles: 13.0-15.0Hz, Beta: 15.0-25.0Hz, Gamma 1: 25.0-40.0Hz, and Gamma 2: 60.0-90.0Hz. * $p < 0.05$, ** $p < 0.01$.

Supplementary table 2. Percentage of changes in F electrodes in FFT power during the first 10 seconds of the odor on-interval with the last 10 seconds of the odor off-interval set to 100%.

	Congruent Odor	Incongruent Odor	Vehicle	*F* (2,30)	*p*
Slow Delta	97.3 ± *0.7*	100.6 ± *0.7*	98.8 ± *0.7*	5.6	0.009**
Delta	104.5 ± *1.3*	97.4 ± *1.4*	101.1 ± *1.2*	7.1	0.003**
Theta	104.0 ± *2.2*	99.1 ± *2.3*	102.5 ± *2.1*	1.2	> 0.20
Alpha	103.2 ± *3.5*	101.1 ± *3.7*	105.9 ± *3.4*	0.5	> 0.60
Slow Spindles	102.7 ± *4.6*	102.2 ± *4.8*	103.5 ± *4.4*	0.02	> 0.90
Fast Spindles	110.4 ± *5.1*	96.9 ± *5.4*	101.9 ± *4.9*	1.7	0.20
Beta	96.7 ± *3.8*	100.0 ± *4.0*	100.6 ± *3.6*	0.3	> 0.70
Gamma 1	90.8 ± *8.0*	98.1 ± *4.5*	88.8 ± *6.3*	0.5	> 0.50
Gamma 2	91.3 ± *7.6*	95.8 ± *2.8*	85.1 ± *7.1*	0.7	> 0.50

Means ± s.e.m. as well as *F* and *p*-values from one-way ANOVAs are indicated. Frequency bands are defined as followed: Slow Delta: 0.5-1.5Hz, Delta: 1.5-4.5Hz, Theta: 4.5-8.0Hz, Alpha: 8.0-11.0Hz, Slow Spindles: 11.0-13.0Hz, Fast Spindles: 13.0-15.0, Hz Beta: 15.0-25.0Hz, Gamma 1: 25.0-40.0Hz, and Gamma 2: 60.0-90.0Hz. ** $p < 0.01$.

Supplementary table 3. Percentage of changes in C electrodes in FFT power during the first 10 seconds of the odor on-interval with the last 10 seconds of the odor off-interval set to 100%.

	Congruent Odor	Incongruent Odor	Vehicle	*F* *(2,30)*	*p*
Slow Delta	97.8 ± *0.8*	100.8 ± *0.9*	99.8 ± *0.8*	3.1	0.06
Delta	104.4 ± *1.5*	97.5 ± *1.6*	100.1 ± *1.5*	4.9	0.01*
Theta	103.4 ± *2.4*	100.2 ± *2.5*	100.3 ± *2.3*	0.6	> 0.50
Alpha	101.1 ± *3.7*	101.7 ± *3.9*	102.5 ± *3.6*	0.04	> 0.90
Slow Spindles	103.0 ± *3.8*	100.9 ± *4.0*	100.6 ± *3.7*	0.1	> 0.80
Fast Spindles	111.6 ± *4.6*	95.2 ± *4.8*	99.7 ± *4.4*	3.3	0.049*
Beta	98.9 ± *3.3*	101.0 ± *3.5*	98.3 ± *3.2*	0.2	> 0.80
Gamma 1	94.0 ± *4.1*	99.7 ± *3.9*	84.7 ± *7.9*	1.7	> 0.20
Gamma 2	92.1 ± *4.2*	98.9 ± *1.1*	84.8 ± *7.1*	1.9	0.17

Means ± s.e.m. as well as *F* and *p*-values from one-way ANOVAs are indicated. Frequency bands are defined as followed: Slow Delta: 0.5-1.5Hz, Delta: 1.5-4.5Hz, Theta: 4.5-8.0Hz, Alpha: 8.0-11.0Hz, Slow Spindles: 11.0-13.0Hz, Fast Spindles: 13.0-15.0Hz, Beta: 15.0-25.0Hz, Gamma 1: 25.0-40.0Hz, and Gamma 2: 60.0-90.0Hz. * $p < 0.05$.

Supplementary table 4. Percentage of changes in P electrodes in FFT power during the first ten seconds of the odor on-interval with the last ten seconds of the odor off-interval set to 100%.

	Congruent Odor	Incongruent Odor	Vehicle	*F* *(2,30)*	*p*
Slow Delta	98.1 ± *1.0*	100.8 ± *1.0*	99.7 ± *0.9*	1.9	0.17
Delta	104.7 ± *1.7*	97.4 ± *1.8*	99.7 ± *1.7*	4.6	0.02*
Theta	103.3 ± *2.6*	98.7 ± *2.7*	98.7 ± *2.4*	1.1	> 0.30
Alpha	99.9 ± *4.2*	98.9 ± *4.4*	100.5 ± *4.0*	0.04	> 0.90
Slow Spindles	104.2 ± *3.8*	99.4 ± *4.0*	100.9 ± *3.7*	0.4	> 0.60
Fast Spindles	111.3 ± *4.2*	94.3 ± *4.4*	98.9 ± *4.0*	4.2	0.03*
Beta	100.5 ± *3.1*	98.3 ± *3.3*	101.5 ± *3.0*	0.3	> 0.80
Gamma 1	98.3 ± *4.4*	98.9 ± *3.8*	95.0 ± *5.3*	0.2	> 0.80
Gamma 2	89.2 ± *8.1*	95.0 ± *5.0*	83.9 ± *7.1*	0.6	> 0.50

Means ± s.e.m. as well as *F* and *p*-values from one-way ANOVAs are indicated. Frequency bands are defined as followed: Slow Delta: 0.5-1.5Hz, Delta: 1.5-4.5Hz, Theta: 4.5-8.0Hz, Alpha: 8.0-11.0Hz, Slow Spindles: 11.0-13.0Hz, Fast Spindles: 13.0-15.0Hz, Beta: 15.0-25.0Hz, Gamma 1, 25.0-40.0Hz, and Gamma 2, 60.0-90.0Hz. * $p < 0.05$.

Supplementary table 5. Fast spindle density for all channels.

Channel	Congruent Odor	Incongruent Odor	Vehicle	$F_{(2,29)}$	p
F3	90.9 ± *21.7*	123.4 ± *27.3*	146.0 ± *43.1*	0.8	> 0.4
F4	103.1 ± *26.5*	190.6 ± *38.8*	122.0 ± *28.8*	2.1	0.14
C3	124.4 ± *13.0*	100.1 ± *20.7*	161.8 ± *25.4*	2.3	0.12
C4	150.0 ± *15.4*	133.3 ± *44.0*	162.0 ± *33.5*	0.2	> 0.80
P3	134.5 ± *17.1*	116.0 ± *24.1*	120.9 ± *12.9*	0.3	> 0.70
P4	118.4 ± *10.5*	91.3 ± *17.5*	146.4 ± *29.2*	1.7	0.20

Means ± s.e.m. as well as *F* and *p*-values from one-way ANOVAs are indicated.

Supplementary table 6. Percentage of changes in FFT power during the entire stimulation interval.

Band	Congruent Odor	Incongruent Odor	Vehicle	*F* (2,30)	*p*
Slow Delta	99.2 ± *0.7*	100.3 ± *0.4*	99.3 ± *0.4*	1.4	0.26
Delta	101.0 ± *0.8*	98.6 ± *0.5*	101.2 ± *0.7*	4.6	0.02*
Fast Spindle	103.0 ± *2.8*	92.9 ± *3.1*	99.3 ± *1.8*	3.7	0.04*

Data are retrieved form frontal (slow delta and delta band) and parietal (fast spindle band) electrodes during the whole 30s of the odor on-interval compared to the whole 30s of the preceding odor off-interval set to 100%. Data are means ± s.e.m. Right columns indicate *F* and *p*-values for one-way ANOVA. * indicating a *p*-value < 0.05, ** $p < 0.01$.

Supplementary table 7. Mean values of EEG arousals for odor on- and odor off-intervals as well as their absolute difference.

	Congruent Odor	Incongruent Odor	Vehicle	$F_{(2,30)}$	p
10-s intervals					
Odor on	1.45 ± *0.31*	0.90 ± *0.28*	1.33 ± *0.40*	0.7	> 0.50
Odor off	1.82 ± *0.58*	1.10 ± *0.28*	1.17 ± *0.42*	0.8	> 0.40
Difference on/off	-0.36 ± *0.60*	-0.20 ± *0.36*	0.17 ± *0.34*	0.4	> 0.60
30-s intervals					
Odor on	5.09 ± *0.93*	2.70 ± *0.47*	4.33 ± *0.67*	2.7	0.09
Odor off	5.18 ± *1.42*	3.20 ± *0.57*	4.83 ± *0.87*	1.0	> 0.30
Difference on/off	-0.09 ± *0.68*	-0.50 ± *0.72*	-0.50 ± *0.65*	0.1	> 0.80

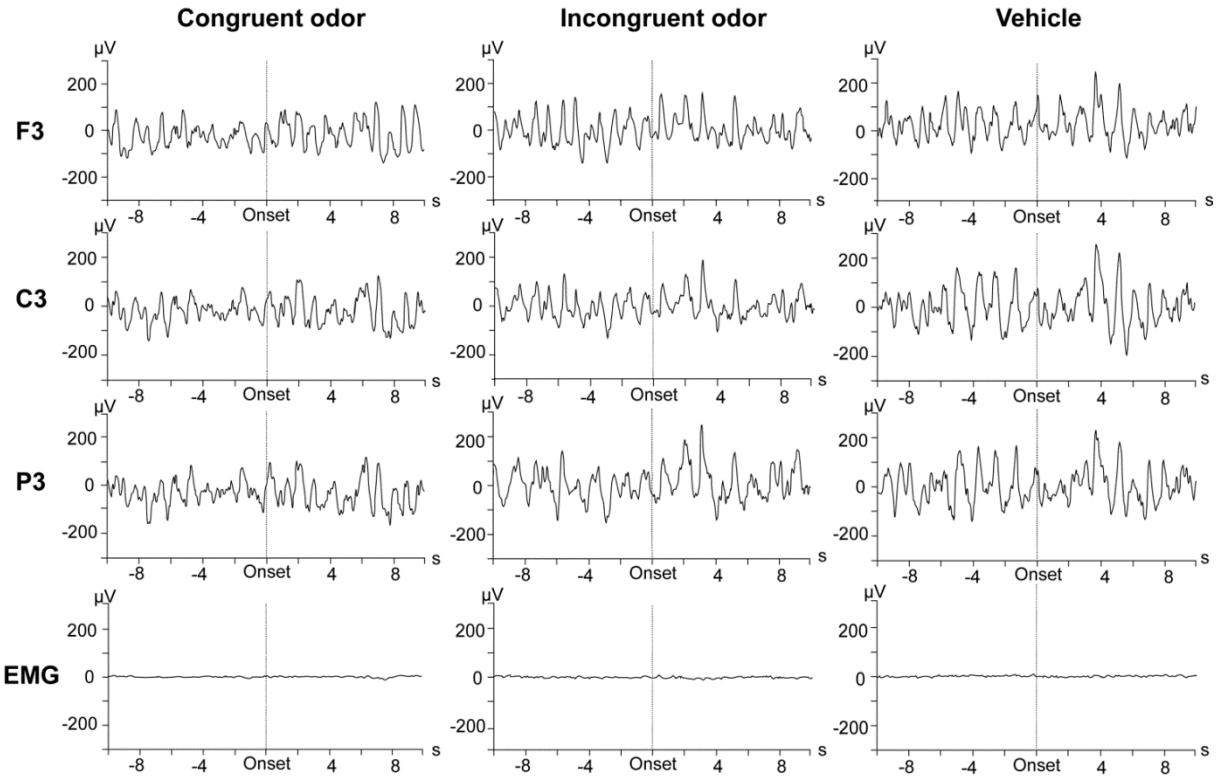
Means ± s.e.m. as well as *F* and *p*-values from one-way ANOVAs are indicated.

Supplementary table 8. Mean values of EEG arousals starting at 16Hz for odor on- and odor off-intervals as well as their absolute difference.

	Congruent Odor	Incongruent Odor	Vehicle	$F_{(2,30)}$	p
10-s intervals					
Odor on	0.64 ± *0.28*	0.30 ± *0.15*	0.17 ± *0.11*	1.6	> 0.20
Odor off	1.00 ± *0.45*	0.80 ± *0.39*	0.92 ± *0.31*	0.07	> 0.90
Difference on/off	-0.36 ± *0.47*	-0.50 ± *0.40*	-0.75 ± *0.30*	0.3	> 0.70
30-s intervals					
Odor on	2.45 ± *0.77*	1.70 ± *0.40*	0.67 ± *0.26*	3.2	0.06
Odor off	2.82 ± *0.84*	3.10 ± *0.89*	2.25 ± *0.48*	0.4	> 0.70
Difference on/off	-0.36 ± *0.61*	-1.40 ± *0.76*	-1.58 ± *0.51*	1.1	> 0.30

Data was high-pass filtered at 16Hz before identification of EEG arousals. Means ± s.e.m. as well as *F* and *p*-values from one-way ANOVAs are indicated.

Supplementary figure 1:



Supplementary Figure 1: Examples of 20s (10s before and 10s after odor or vehicle onset) raw EEG and EMG data for the congruent, incongruent and vehicle condition. Channels F3, C3, P3 and EMG are displayed. Visual raw data inspection suggested that the changes in delta and fast spindle power in the congruent group were not caused by arousal responses.

4.2 Study 2: Neural correlates of olfactory activation during sleep

Planned as: Rihm, J. S., Shanahan, L., Bosch, O.G., Stämpfli, P., Seifritz, E., Gottfried, J.A., & Rasch, B. (2014). Neural correlates of olfactory activation during sleep.

4.2.1 Abstract

Olfactory stimuli are of high evolutionary importance and are thus processed even during sleep. However, evidence suggests that the primary brain area linked to processing odors – the piriform cortex – seems to be hyposensitive to input during the sleep state. Yet because multiple studies have shown that presenting olfactory cues associated with previously learned stimuli during sleep benefits memory consolidation, the access to brain regions involved in memory processing seems given. Here, we investigated whether olfactory stimuli differentially activate brain regions involved in the processing of memories and emotions when they are presented during sleep compared with wakefulness. We presented three odors (one neutral, one negative, one positive) as well as an odorless vehicle in random order while participants slept or remained awake in an fMRI scanner. Our results demonstrate that olfactory stimulation activates the anterior and posterior piriform cortices, as well as amygdala, to a higher extent during wakefulness compared with sleep. Olfactory stimulation did not activate the hippocampus during wakefulness or sleep. Additional analyses in process include the matching of odor onsets to inhalation and fMRI pattern analysis, which should further specify the differences in odor processing during wake and sleep.

4.2.2 Introduction

Odors provide us with important information about our environment. Thus, it is not surprising that olfactory processing during wakefulness is well-studied, and that its unique characteristics are known. For example, when comparing processing in the olfactory system with other sensory modalities, odors are processed ipsilaterally, they bypass the thalamus, and the olfactory bulb is closely connected to brain regions implicated in memory processing (i.e., hippocampus and entorhinal cortex – and emotional processing (i.e., amygdala (Gottfried, 2006).

Interestingly, odors and their valence seem to be processed not only during wakefulness, but also during sleep. This is evidenced by the finding that odors presented during rapid eye movement (REM) sleep are incorporated into dreams (Trotter, Dallas, & Verdone, 1988). Furthermore, presentation of pleasant or unpleasant odors during REM sleep led to congruent emotional valence in dream reports (Schredl et al., 2009). Additional evidence for active odor processing during sleep arises from a recent study demonstrating that it is possible to learn entirely new olfactory-associated information during sleep. Fear conditioning by pairing pleasant and unpleasant odors with different tones during sleep had an influence on the behavior when these tones were re-played during post-conditioning wakefulness: sniff responses were smaller to the tones associated with unpleasant odors compared with pleasant odors (Arzi et al., 2012).

Furthermore, it is possible to enhance memory performance by presenting odors during sleep that were previously associated with learning during wakefulness. For example, when a background odor is present while subjects learn card pair locations, presentation of the same odor during slow-wave sleep (SWS) leads to better memory performance upon waking compared with the presentation of a vehicle (Diekelmann, Büchel, Born, & Rasch, 2011; Rasch, Büchel, Gais, & Born, 2007) or a novel odor (Rihm, Diekelmann, Born, & Rasch, 2014). This declarative memory-enhancing effect of odor presentation during sleep has now been shown in three independent studies (Diekelmann et al., 2011; Rasch et al., 2007; Rihm et al., 2014). Additional studies could show that even behavioral therapy success (Rihm et al., submitted) or creativity (Ritter, Strick, Bos, Van Baaren, & Dijksterhuis, 2012) can be cued by the presentation of a task-associated odor during sleep.

A possible explanation for the memory-enhancing effect of olfactory stimuli was suggested by a rodent study using local field potentials. In this study, rats were fear conditioned by pairing an odor (conditioned stimulus, CS+) with a foot shock (unconditioned stimulus, UCS). The odor was presented again during slow-wave states (SWS) and high-wave states (wake, REM), and odor-evoked responses in power bands in the piriform cortex were compared. The responsiveness of the piriform cortex to odors was significantly reduced during SWS compared with REM sleep or wakefulness (Barnes, Chapuis, Chaudhury, & Wilson, 2011). A possible explanation for this finding is that the piriform cortex goes offline during SWS to reduce external interference, sheltering it from processing novel information. However, the differential processing of olfactory stimuli in sleep could alternatively serve to facilitate access of newly learned olfactory information to regions with piriform projections specific to learning and memory, such as entorhinal cortex, hippocampus, and amygdala.

Until now, the neural correlates involved in olfactory processing during sleep compared with wakefulness have not been examined in humans. To investigate this open question, we conducted an fMRI study using a within-subject design to compare the differences in brain activity while processing olfactory stimuli during sleep versus wakefulness. Odors were presented via an odor delivery mask during wakefulness and sleep while subjects lay in an fMRI scanner. We hypothesized that brain activation during olfactory processing in sleep would be less pronounced in the piriform cortex, but that there may be higher activation in amygdala and hippocampus compared with wakefulness.

4.2.3 Methods

Participants. Forty-two healthy, non-smoking subjects naïve to the experiment protocol participated in the study. Of the 42 participants, 29 were excluded because they were unable to fall asleep in the scanner. Thus, our final sample consisted of thirteen female subjects (mean age: 23.08 ± 2.81 (SD) years; range: 19-29 years). Though we recruited male subjects, they either did not meet our inclusion criteria or failed to fall asleep in the scanner. Participants underwent a sleep and a wake session in counterbalanced order. The sample consisted of data from eight subjects collected at the University of Zurich, Switzerland. A second sample with data from five additional subjects was collected at Northwestern University, USA. Since the experimental procedures and scanner protocols were identical for both samples, and because data were collected in a within-subject design, we analyzes up these two samples together.

Participants were not taking any medication at the time of the study, and reported a normal sleep-wake cycle. According to pre-study interviews, all participants considered themselves to be good sleepers, had not been working night shifts, and did not have any major sleep disturbances for six weeks prior to the experiment. Sleep quality was assessed by the Pittsburgh Sleep Quality Index (PSQI; Buysse, Reynolds, Monk, Berman, & Kupfer, 1989; mean PSQI score: 3.62 ± 0.54). Any participants with nasal infections were excluded on the days of the experiments.

On experimental days, participants were instructed to refrain from taking naps and from ingesting alcohol or caffeinated drinks. Written informed consent was obtained from all subjects prior to participation. The experiment was approved by the Cantonal Ethics Committee of Zurich and by the Northwestern University Institutional Review Board.

Odor delivery and substance. Three highly-distinct olfactory stimuli were utilized in the experiment: a positive odor (citral; Sigma-Aldrich, Germany), a neutral odor (beta-ionone; Sigma-Aldrich, Germany) and a negative odor (4-methylpentanoic acid; Sigma-Aldrich, Germany). The odors were diluted in odorless mineral oil (1,2-propanediol; Sigma-Aldrich, Germany) at a concentration of 1:100 (citral), 5:100 (4-methylpentanoic acid) and 2:100 (beta-ionone). We matched the intensities of these concentrations based on pilot studies. The odorless mineral oil served as control stimulus. Odors were delivered via a 12-channel computer-controlled olfactometer designed after Lorig (2000). Room air was filtered before entering the system, and airflow was held constant at 3 l/min. To avoid tactile or thermal shifts associated with odor onset, half of the air stream was presented continuously to the subject, and the other half alternated between room air and vehicle or odor presentation. The olfactometer was placed outside of the scanner room. The subject received the odor via a small nasal mask or, in the second sample, via a thing odor delivery tube under the nose, which assured constant stimulation, but permitted normal breathing. In the first sample, the glass bottles containing odorants were placed beside the scanner, thus allowing rapid odor onset and offset times of 500ms. In the second sample, the glass bottles containing the odorants were placed outside the scanner, which prolonged odor onset and offset times to 2000ms. For fMRI analysis, these delays were taken into account by adding them to the time point when the odor valves were opened.

Design and procedure. All participants underwent two experimental conditions during which they either laid awake or slept in an MRI scanner. The procedures for both sessions were identical: participants breathed normally while a positive, a negative, a neutral odor, and an odorless vehicle were presented in random order for 4s each with a 20s ITI to avoid habituation. For the wake group, odors were presented 18 times each, but during the sleep session, the number of presentations could not be as standardized as during wakefulness, since number of odor deliveries was dependent on the amount of time subjects remained asleep. However, we also had to exclude some wake trials if off-line EEG scoring indicated that participants dozed off during their wake session. After exclusion, the mean number of odor presentations during sleep (77.69 ± 9.94) was slightly higher than during wakefulness (62.23 ± 3.90), but the difference did not reach statistical significance ($t(12) = 1.67$, $p = 0.12$). In order to keep the wake and the sleep session as similar to each other as possible, we did not cue subjects to sniff at odor presentations during the wake session, since this would not have been possible during sleep. The wake session started at 5p.m. and participants lay in the

scanner from 6.30p.m. until 7.30p.m. In the sleep session, participants came at 7p.m. and lay in the scanner until approximately 9pm. Duration of the sleep session was dependent on the amount of time subjects needed to fall asleep and were able to sleep, but it was never longer than 1.5hrs. During the night prior to the sleep session, subjects limited their sleep to three hours maximum. This was monitored by an Actiwatch that computes sleep times based on light and movement information, or by a Fitbit in the second sample. Visual inspection of the monitoring data confirmed that all of the subjects slept for three hours or less the night prior to participation in the sleep session.

At the beginning of each experimental session, subjects completed questionnaires concerning personal information and sleep parameters. Thereafter, the EEG cap was attached, impedances were measured, and participants entered the fMRI scanner. While lying in the scanner, subjects smelled each of the three different odors and the odorless vehicle eighteen times in the wake group a randomized order. Odor presentation started immediately in the wake condition. In contrast, during the sleep condition, EEG was monitored and odors were not administered until stable sleep stage 2 was reached. Stimulus presentation was stopped whenever signs of arousal or awakening appeared in the EEG. After the scan session in the wake condition, subjects rated the three odors according to pleasantness, arousal, and familiarity, and performed an odor detection test with each of the three odors.

Polysomnography. Sleep was recorded by standard polysomnography using a 32-channel Brain Vision MR-compatible EEG cap, and data was sampled at 5000Hz. In the second part of the sample, sleep was recorded using a 64-channel Neuroscan MR-compatible EEG cap and data was sampled at 10000HZ. Impedances were kept below 20 kΩ.

In addition to the online identification of sleep stages, polysomnographic recordings were scored offline by two independent raters according to standard criteria (Iber et al., 2008). The sleep stage scored was N2. A fine-grained differentiation between N1, N2 and N3 was not possible due to quality limitations in the EEG data because of gradient artifacts and their correction.

fMRI data acquisition. Measurements were performed on a Philips Achieva TX 3 Tesla whole-body magnetic resonance unit equipped with an 8-channel head array and a Siemens 3 Tesla with a 32-channel head coil. Subjects were instructed to lie still in the scanner with their eyes closed during data acquisition. Functional images were collected using a sensitivity-encoded single-shot echo-planar sequence (TE = 20 ms; field of view = 22 cm, flip angle =

82°; acquisition matrix = 88 x 85, interpolated to 96 x 96, voxel size = 2.50 x 2.50 x 2.50 mm, reconstructed to 2.29 x 2.29 x 2.5 and sensitivity-encoded acceleration factor R = 2.5) sensitive to BOLD contrast (T2* weighting). Using a midsagittal scout image, 42 contiguous axial slices were recorded in an interleaved manner and placed along the anterior-posterior commissure plane covering the entire brain with a repetition time of 2500ms.

fMRI data preprocessing. Analysis of fMRI data was performed using SPM8 (Wellcome Trust Centre for Neuroimaging, London). For the sleep session, sleep scoring was used to identify the scans where participants were asleep during odor delivery. If participants moved or woke up during sleep between two or more sleep blocks with odor presentations, we treated the data as multiple sessions and took this into account during preprocessing. For the wake session, sleep scoring was also performed to verify the wake status of the participants during these scans. Images were slice-timed to the onset of the middle slice, realigned to the first scan to correct for head movements, spatially normalized to a standard EPI image, and spatially smoothened using an 8-mm full-with at half maximum Gaussian kernel. Smoothing was not performed for multivariate pattern analysis in order to preserve individual voxel information.

fMRI univariate analysis. At the first level, we specified odor onsets by adding the time from odor presentation to odor perception (i.e. 500ms for the first sample and 2000ms for the second sample) to account for time delay between odor presentation and perception. After estimating the first level model, we constructed a within-subjects model for the wake and the sleep session of each subject based on regressors for each of the four stimuli (citral, beta-ionone, 4 MPA, odorless vehicle). SPM images were thresholded at $p < 0.001$, small volume corrected (SVC), with no threshold in voxel cluster size. Since we had clear hypotheses about the brain regions that should be differentially involved in odor processing during sleep compared with wakefulness, we focused on the following regions of interest (ROIs): bilateral anterior piriform cortex (APC), bilateral posterior piriform cortex (PPC), bilateral amygdala, and bilateral hippocampus. ROIs were drawn manually in MRIcron software based on the Atlas of the Human Brain (Mai, Assheuer, & Paxinos, 1997).

4.2.4 Results

In order to compare brain activation between sleep and wakefulness in general and in emotional odor processing, we focused on the following two contrasts: (i) the comparison

between all three odors (positive, neutral, negative) versus the odorless vehicle in sleep compared to wakefulness, and (ii) the comparison between the emotional odors (positive and negative) versus the odorless vehicle in sleep and wakefulness.

Anterior and posterior piriform cortex. In line with our hypothesis, there was greater activation of APC and PPC during wakefulness than during sleep (see table 1). This was the case when we compared all three odors with the odorless vehicle (APC: $Z = 3.73$, $P_{SVC} < 0.001$; PPC: $Z_{max} = 3.95$, $P_{SVC} < 0.001$; see figure 1), as well as when we compared only the two emotional odors with the odorless vehicle (APC: $Z_{max} = 4.14$, $P_{SVC} < 0.001$; PPC: $Z_{max} = 4.42$, $P_{SVC} < 0.001$; see figure 2).

Amygdala. Contrary to our hypothesis, amygdala activation in response to all three odors ($Z = 3.93$, $P_{SVC} < 0.001$; see figure 1) and emotional odors ($Z_{max} = 4.03$, $P_{SVC} < 0.001$; see figure 2) compared to the odorless vehicle resulted in higher activation during wakefulness compared with sleep (see table 1).

Table 1. Brain regions differentially activated for wake compared with sleep.

		MNI coordinates (mm)					
		x	y	z	*Z* score	P_{SVC}	Cluster size
All odors > vehicle & wake > sleep							
APC	l. APC	-24	-56	-22	3.73	< 0.001	1
PPC	l. PPC	-22	2	-22	3.95	< 0.001	23
	r. PPC	30	6	-24	3.37	< 0.001	3
amygdala	l. amygdala	-22	0	-24	3.93	< 0.001	11
Emo. odors > vehicle & wake > sleep							
APC	l. APC	-26	10	-20	4.14	< 0.001	8
	r. APC	34	14	-14	3.52	< 0.001	1
PPC	l. PPC	-22	2	-22	4.42	< 0.001	27
	r. PPC	30	6	-24	3.79	< 0.001	11
amygdala	l. amygdala	-22	0	-24	4.03	< 0.001	17
	r. amygdala	22	0	-24	3.48	< 0.001	4
	r. amygdala	28	0	-26	3.18	< 0.001	1

Brain regions that are more active during wakefulness than during sleep when comparing all odors with vehicle, and only the emotional odors with vehicle. L: left, r:right, APC: anterior piriform cortex, PPC: posterior piriform cortex, SVC: small volume corrected.

Hippocampus. Again, contrary to our hypothesis, hippocampal we did not find any difference in hippocampal activation between the wakefulness and sleep, either for all three odors or for the emotional odors.

One possible explanation for this finding could be that hippocampal activation is at the same level during odor perception during sleep and wakefulness, and thus did not reveal any significant differences in our contrast analyses. Therefore, we examined if our two contrasts of interest elicit similar hippocampal activation in sleep and wake alone, but neither the comparison between all odors and vehicle, nor the comparison between emotional odors and vehicle activated the hippocampus during wake or sleep alone.

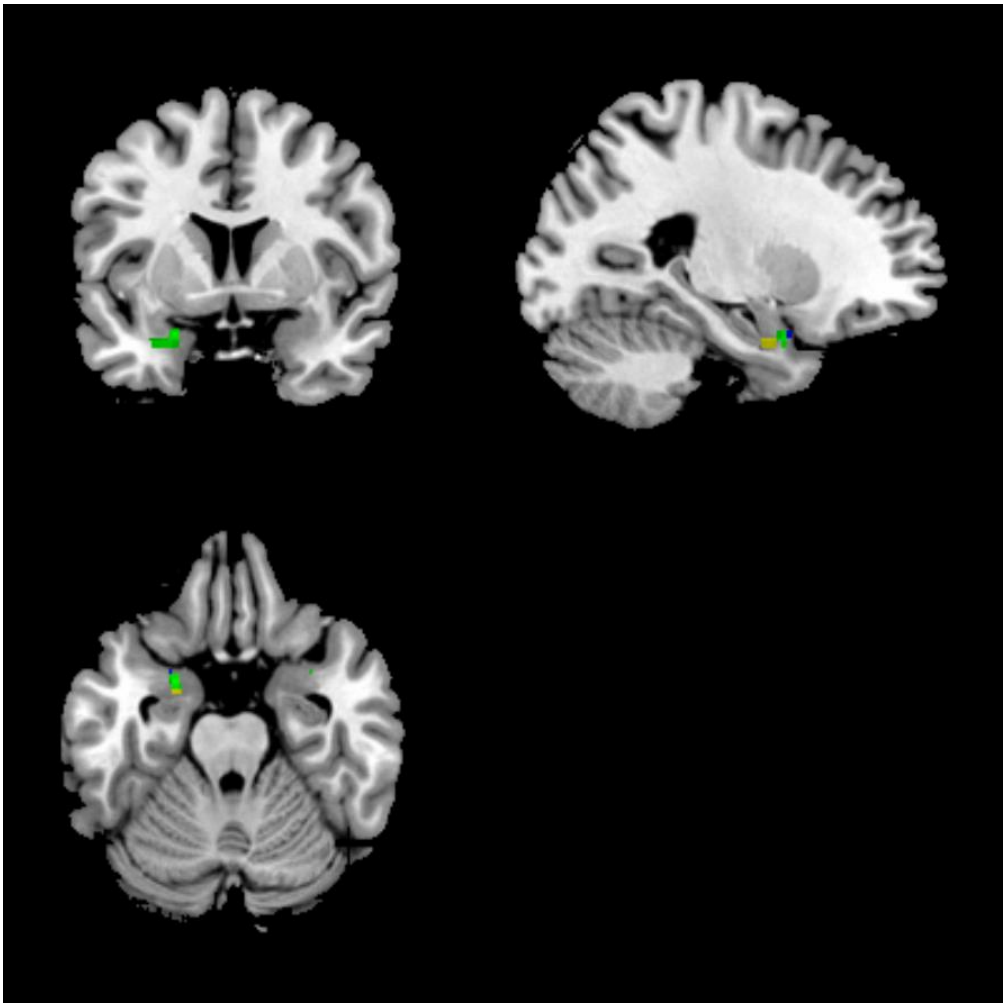


Figure 1: Brain activation higher for wakefulness compared with sleep in response to all three odors compared with vehicle. The colors of the voxel clusters illustrate anterior piriform cortex activation in blue, posterior piriform cortex activation in green, and amygdala activation in yellow (MNI coordinates of the image: x= -24, y= 5, z= -23).

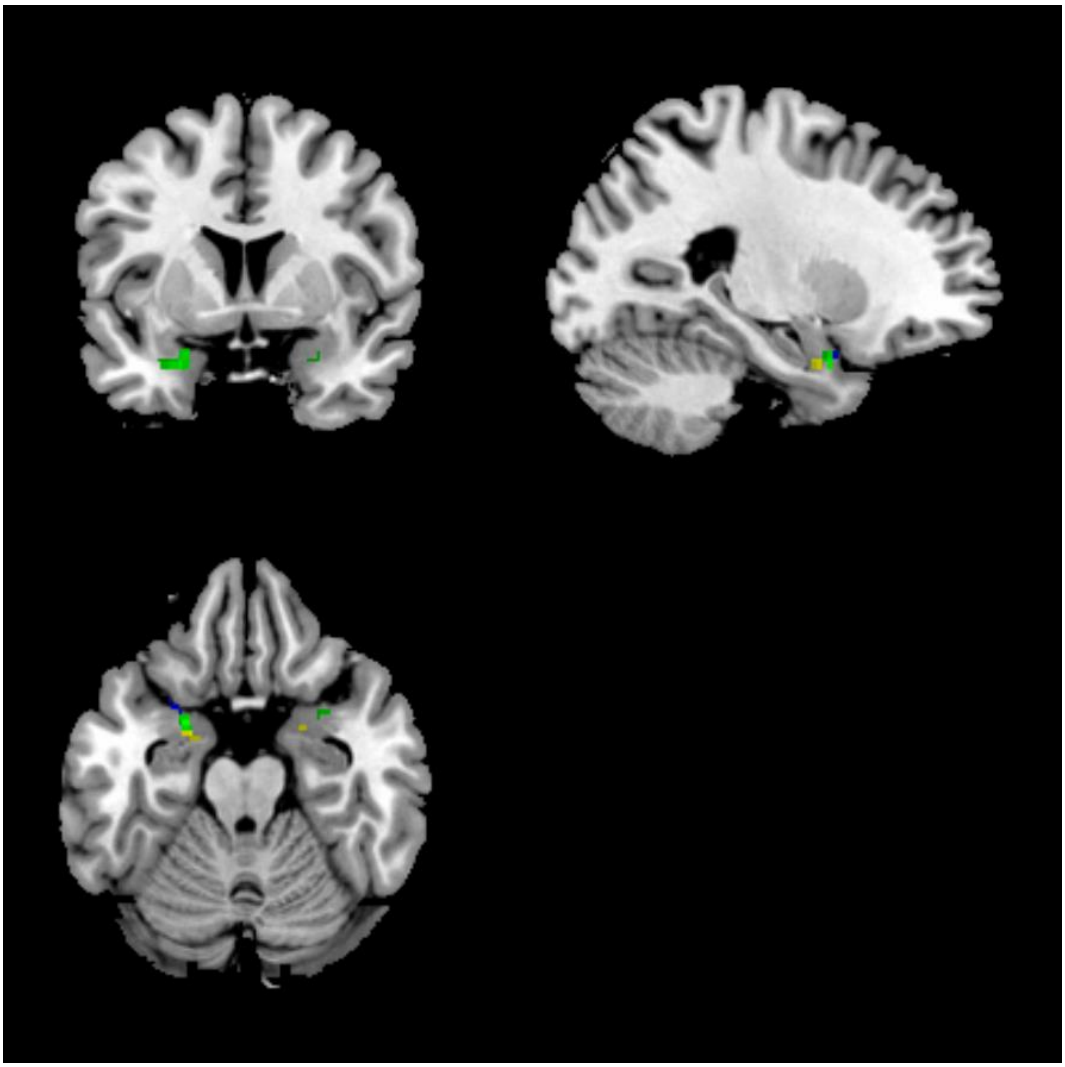


Figure 2: Brain activation higher for wakefulness compared with sleep in response to the emotional odors compared with vehicle. The colors of the voxel clusters illustrate anterior piriform cortex activation in blue, posterior piriform cortex activation in green, and amygdala activation in yellow (MNI coordinates of the image: x= -24, y= 5, z= -21).

4.2.5 Discussion

Our results show that odor processing during wakefulness activates the piriform cortex and the amygdala to a higher extend than odor processing during sleep. Neither the hippocampus, nor the amygdala was more activated during sleep compared with wake when mixed or emotional odor stimuli were presented.

The hyporesponsiveness of the human piriform cortex during sleep is not surprising and was already shown in a previous rodent study (Barnes et al., 2011). In this study, local field potentials from rats were recorded during odor stimulation during sleep and wakefulness. The signals in the piriform cortex in response to stimulation during sleep were much weaker

than those during wake. Here we confirm these findings for the first time in human subjects, since we also found evidence of significantly higher activation in APC and PPC during wakefulness than during sleep.

The different levels of piriform cortex activation during olfactory processing in sleep and wakefulness leads to the assumption that other areas – most likely areas that have direct projections to the piriform cortex – are involved in odor processing during sleep. Candidates for such a mechanism are the hippocampus and the amygdala, which are important in memory and emotional processing, respectively. However, our data does not provide evidence for an important role of these structures during olfactory processing in sleep.

Yet, the absence of superior activation in the hippocampus during sleep is not so surprising, since we did not associate the odors with a memory task. Previous studies that could show hippocampal activation during presentation of an odor in sleep linked this odor with a pre-sleep learning task (Rasch et al., 2007). Thus, it is nevertheless possible that the odors have a facilitated access to brain regions associated with memory and emotional processing, but that this access only results in an activation of these regions if the odor was previously linked with information that is stored in these regions.

As opposed to perception of acoustic stimulation, the perception of odors depends on inhalation. Usually, studies investigating brain activation in response to odors cue participants to sniff when they present an odor to exclude breathing as a confounding variable. We intentionally decided against such a paradigm, since we wanted the sleep and wake session to be as similar as possible. But especially during sleep, inhalation is typically slower and shallower. It could be that some odors were only perceived for a shorter time, or that odors delivered for 4s were not perceived at all for slow breathers. Therefore, the last step in the analysis will be to match the onset times not only to the release of the odor plus the time hypothetically needed for odor perception, but to the onset of inhalation after the odor release. Furthermore, another possible analysis could be to take include the individual inhalation amplitudes after each odor presentation as a covariate in our second level.

Pattern analysis possibly can help to detect differences not seen in univariate fMRI analysis, because it takes into account pattern of activation which involve voxels that are not significantly activated in a ROI analysis but might still be differentially activated during sleep compared with wakefulness.

4.2.6 References

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4.3 Study 3: Replay of conditioned stimuli during late REM and stage N2 sleep influences affective tone rather than emotional memory strength

Published as: Rihm, J. S., & Rasch, B. (2015). Replay of conditioned stimuli during late REM and stage N2 sleep influences affective tone rather than emotional memory strength. *Neurobiology of Learning and Memory, 122*, 142-151.

4.3.1 Abstract

Emotional memories are reprocessed during sleep, and it is widely assumed that this reprocessing occurs mainly during rapid eye movement (REM) sleep. In support for this notion, vivid emotional dreams occur mainly during REM sleep, and several studies have reported emotional memory enhancement to be associated with REM sleep or REM sleep-related parameters. However, it is still unknown whether reactivation of emotional memories during REM sleep strengthens emotional memories. Here, we tested whether re-presentation of emotionally learned stimuli during REM sleep enhances emotional memory. In a splitnight design, participants underwent Pavlovian conditioning after the first half of the night. Neutral sounds served as conditioned stimuli (CS) and were either paired with a negative odor (CS+) or an odorless vehicle (CS-). During sound replay in subsequent late REM or N2 sleep, half of the CS+ and half of the CS- were presented again. In contrast to our hypothesis, replay during sleep did not affect emotional memory as measured by the differentiation between CS+ and CS- in expectancy, arousal and valence ratings. However, replay unspecifically decreased subjective arousal ratings of both emotional and neutral sounds and increased positive valence ratings also for both CS+ and CS- sounds, respectively. These effects were slightly more pronounced for replay during REM sleep. Our results suggest that re-exposure to previously conditioned stimuli during late sleep does not affect emotional memory strength, but rather influences the affective tone of both emotional and neutral memories.

4.3.2 Introduction

Emotional events undergo preferential memory consolidation compared with neutral events (Cahill & McGaugh, 1998; McGaugh, 2006). In a laboratory setting, emotional learning is

usually investigated with Pavlovian conditioning (Pavlov, 1927). In this procedure, an initially neutral conditioned stimulus (CS) is repeatedly presented with an aversive unconditioned stimulus (UCS). Importantly, different time contingencies between the CS and the UCS involve different brain structures: learning the association by trace conditioning with a time delay after the offset of the CS and the onset of the UCS is hippocampus-dependent (Solomon, Vander Schaaf, Thompson, & Weisz, 1986), whereas delay conditioning with an overlapping presentation of the UCS at the end of the CS presentation relies mostly on the amygdala (Fanselow & LeDoux, 1999; Maren, 2001).

Sleep is assumed to play an important role in emotional memory re-processing (Hu, Stylos-Allan, & Walker, 2006; Wagner, Gais, & Born, 2001; for a review see Payne & Kensinger, 2010). In particular, it is widely assumed that rapid eye movement (REM) sleep, earlier termed as dream sleep, is critically involved in this process. Possibly related to the notion that vivid and emotional dreams mostly occur after awakening from REM sleep (Hobson, Pace-Schott, & Stickgold, 2000), many psychoanalytic approaches assume that dream sleep-related dreams are critically related to reprocessing of previously encountered emotional events (Freud, 1900). In addition, a high number of studies in rodents using emotional learning tasks like Pavlovian conditioning or active avoidance tasks have revealed strong evidence for a role of REM sleep in emotional memory consolidation: In these studies, REM sleep increases are frequently observed after learning during specific time windows and correlated with learning success, while suppressing REM during these times impaired learning (Smith, 1985, 1996 for reviews and meta-analyses). As a possible underlying mechanism, ponto-geniculo-occipital waves occurring during REM sleep as well as reactivations on the amygdala and hippocampus level have been proposed (Datta & O'Malley, 2013; Hennevin, Huetz, & Edeline, 2007). In fact, in rodents, patterns of hippocampal memory reactivation have been observed during REM sleep (Louie & Wilson, 2001; Poe, Nitz, McNaughton, & Barnes, 2000), and presentations of memory cues during REM sleep activate hippocampal neurons (Maho & Bloch, 1992) and increase avoidance responses to previously conditioned stimuli (Hars, Hennevin, & Pasques, 1985).

In addition to the strong evidence for the role of REM sleep in the consolidation of emotional memories in rodents, also human studies provide support for this notion. For example in split-night paradigms, emotional memory for pictures and stories was particularly enhanced after a period of late, REM sleep-rich sleep, but not after periods of early slow-wave sleep (SWS)-rich sleep (Groch, Wilhelm, Diekelmann, & Born, 2013; Wagner, Fischer, & Born, 2002; Wagner et al., 2001). Furthermore, the deprivation of REM sleep led to less

emotional-laden memory of stories (Greenberg, Pearlman, Schwartz, & Grossman, 1983) and changes in REM sleep are often involved in disorders affecting emotional processing (Tsuno, Besset, & Ritchie, 2005). In addition, several studies have reported positive correlations with REM sleep-related parameters and memory for emotional information (Baran, Pace-Schott, Ericson, & Spencer, 2012; Menz et al., 2013; Nishida, Pearsall, Buckner, & Walker, 2009; Payne, Chambers, & Kensinger, 2012). According to a recent theoretical account, emotional memories are reactivated during REM sleep by covert amygdaloid reactivations, causing a strengthening of the declarative, informational content of the memory and a decrease of the emotional reactivity to this memory (Van der Helm et al., 2011; Walker & van der Helm, 2009, although see Baran et al., 2012; see Rasch & Born, 2013 for a review).

While an association between REM sleep and emotional memory appears to be well-established in rodents, experimental evidence for a critical role of emotional memory reactivation during REM sleep for consolidation processes is still scarce in humans. Induced memory reactivation during REM sleep has been shown to improve performance in complex logical tasks (Smith & Weeden, 1990) as well as generalization of sound-face associations (Sterpenich et al., 2014), while no behavioral effects of targeted memory reactivation with respect to the emotionality of the associated faces (i.e., negative vs. neutral) were reported in the latter study. For neutral declarative memory tasks, targeted memory reactivation during REM sleep is ineffective (Cordi, Diekelmann, Born, & Rasch, 2014; Rasch, Büchel, Gais, & Born, 2007). In fact, recent studies revealed evidence that emotional memories could be influenced by reactivation in SWS during midday naps (Cairney, Durrant, Hulleman, & Lewis, 2014; Hauner, Howard, Zelano, & Gottfried, 2013), but these studies did not include a REM sleep condition. Thus, it still remains an open question if reexposure to emotional memories during REM sleep can affect emotional memory performance after sleep in humans.

To test this question, we used a between-subject, split-night design where participants slept during the first half of the night, were awoken to undergo hippocampus-independent Pavlovian delay conditioning after the first half and slept again during the second half. During conditioning learning, half of the neutral sounds were associated with a negative odor (UCS) and the other half with an odorless vehicle, resulting in CS+ (sounds followed by the odor) and CS- (sounds followed by the odorless vehicle). During subsequent sleep in the second half of the night, half of the sounds that were previously learned as negative (CS+) and half of the sounds that were previously learned as neutral (CS-) were replayed without the odor or the odorless vehicle, respectively. The target sleep stages for sound re-presentation differed

between two groups – REM sleep or N2 sleep as control. Recall took place 36 h later, after a recovery night. We hypothesized that replay of the CS+ during REM sleep will result in enhanced memory for this association compared with replay during N2 sleep, expressed in higher odor expectancy, higher physiological reactivity, and higher subjective affective reactivity.

4.3.3 Methods

Participants. Thirty-five healthy, non-smoking women naïve to the experimental protocol participated in the study. Participants were randomly assigned to two groups, depending on the sleep stage during which replay took place: ‘‘REM sleep replay group’’ (N = 19) and ‘‘N2 sleep replay group’’ (N = 16). As exclusion criterion, we defined a difference value between all CS+ and all CS- over the whole course of learning (80 trials total) of 0.5. Based on this differentiation score, data from five subjects had to be excluded. This resulted in 30 participants included in the final analysis (REM replay group: N = 16, N2 replay group: N = 14). Age distribution was highly comparable between sleep replay groups (mean age: 23.30 ± 2.72 years (SD); range: 18–32 years; $t(28) = 0.291$, $p > 0.70$). Participants were in good physical and mental health according to a routine examination: They did not take any psychologically active medication at the time of and one month prior to the experiment, and reported a normal sleep–wake cycle. They had not been on night shift, did not experience long distance flights, did not have any major sleep disturbances during eight weeks prior to the experiment, and had a good sleep quality according to the Pittsburgh Sleep Quality Index (PSQI; Buysse, Reynolds, Monk, Berman, & Kupfer, 1989) (mean PSQI score: 3.50 ± 0.28; no differences between groups: $t(28) = -0.94$, $p > 0.30$). They had normal olfactory and auditory functions. Any nasal infections were excluded on the day of the experiment. Participants underwent a UCS test during which they had to rate the valence of the odor used for aversive conditioning and another odor as distractor on a 9-point scale (1–3 negative, 4–6 neutral, 7–9 positive). They were only included if they rated the UCS odor as negative (mean rating: 2.24 ± 0.77; no significant group difference: $t(28) = 0.53$, $p > 0.50$). On the experimental day, general olfactory performance was tested using the ‘‘Sniffin’ Sticks’’ inventory (Burghart, Germany). The general ability to distinguish between twelve odors (mean: 11.10 ± 0.15) and olfactory thresholds (mean: 5.52 ± 0.35) was comparable between groups (both $p > 0.30$). At the beginning of the main experiment, subjects received the information that the learning task during the night will consist of odors and sounds and that

some sounds will be presented also during sleep at a volume which will not disturb their sleep. Subjects were habituated to the experimental setting by spending an adaptation night in the sleep laboratory under experimental conditions. On experimental days, participants were instructed to get up at 7.00 a.m., not to take any naps and not to ingest alcohol or, after 3.00 p.m., caffeine-containing drinks. All subjects gave written informed consent prior to participation and received monetary compensation after the last session. The experiment was approved by the Cantonal Ethics Commission of Zurich.

Design and procedure. At least two days after fulfilling the UCS odor rating and the adaptation night, participants underwent the emotional learning task. We used a split-night paradigm to separate effects of the second, REM sleep-rich half of the night from the first, SWS-rich half of the night. The session started at 8 p.m. with the application of the electrode cap for polysomnography (PSG) recordings. Thereafter, subjects underwent the olfactory performance tests to control for possible differences between groups. Participants went to bed at around 10 p.m., and after we detected deeper, undisrupted N2 sleep, we let them sleep for around 3 more hours, resulting in an average sleep time of 3.5 h during the first half of the night (209.5 min). They were awoken at 1.30 a.m. to perform the emotional learning task. To avoid possible effects of sleep inertia, participants were asked to walk around for 30 min prior to the task. After completion of the task they went to sleep again at 2.30 a.m. The presentation of auditory stimuli occurred 5 min after detection of subsequent REM or N2 sleep in the online EEG. It was continued as long as subjects stayed in this sleep stage and restarted every time the sleep stage occurred again. Sounds were presented at 45 dB (as tested in a pilot study) with loudspeakers placed in 1 m distance to the left and right behind the bed. Please note that variations in sound pressure level can occur during sound presentation with loudspeakers due to pillow, duvet, and head positioning. Participants were awoken at 6.30 a.m. and allowed to pursue normal daytime activities. To provide participants the opportunity to recover from the stressful split-night paradigm, the night between the experimental night and recall served as recovery night. Recall took place in the late evening after the recovery night, at 8 p.m., to control for circadian influences of time of day on physiological parameters, cortisol, and memory (see Fig. 1 for a procedure overview).

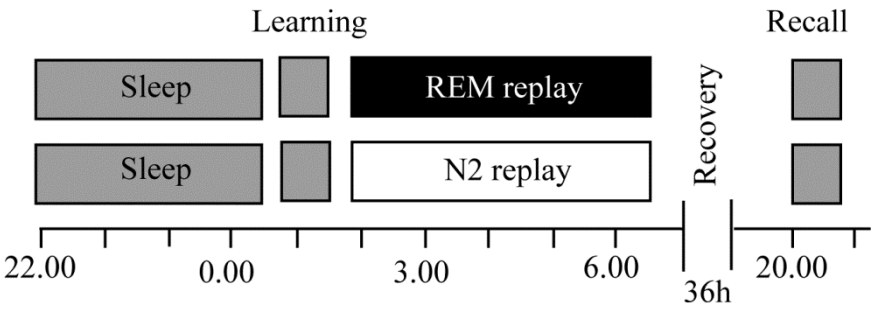

Figure 1: Procedure of the study. Participants of both groups slept the first three hours of the nigth and learned the emotional conditioning task after that. During the second half of the night, half of the CS+ and CS- stimuli were presented again during REM or N2 sleep. In the morning, subjects went home, spent a recovery night at home and came in the evening of the next day for recall the recall task.

Pavlovian conditioning and recall. Conditioning took place on day one of the experiment. Eight normalized neutral sounds of the International Affective Digitized Sound system (IADS; Bradley & Lang, 2007) with a length of 6 s each were presented (typewriter, train, steps, helicopter, floating water, fan, crowd sound, lawn mower). Normative valence (mean: 4.99 ± 0.31 (SD)) and arousal (4.86 ± 0.51 (SD)) ratings (Bradley & Lang, 2007) for the eight sounds on a 9-point scale were chosen according to neutral values and small standard deviations. The assignment to CS+/CS- and replayed/non-replayed sounds was not fixed, but we pseudorandomized the eight sounds in a counterbalanced order, such that every sound was used as CS+ and CS-, and replayed as well as not replayed over the course of the experiment.

At the beginning, we induced a habituation phase during which every sound was presented once and subjects had to indicate the valence and the arousal of the sounds (first arousal/valence rating). During learning, the sounds were presented again 10 times each, and after each of the sound presentations participants rated their odor expectancy. After 1 s, participants had to indicate their odor expectancy on a 9-point scale (1 = not at all, 5 = I do not know, 9 = absolutely certain). Subjects were instructed to rate ‘‘I do not know’’ if they did not yet know and could thus not predict if an odor followed. This screen with the rating scale was displayed for 2 s. After these 3 s the odor (after the CS+ sounds) or the vehicle (after the CS- sounds) were released. If participants failed to give their odor expectancy rating within the 2 s, they received feedback that they answered too slowly to avoid relearning. As a consequence, the odor was not presented, the sound was stopped and the same trial started again. Subjects were instructed about this procedure and the particular trials were excluded from the analysis. The intertrial–interval was randomized between 13 and 17 s.

Each sound was presented 10 times. Since partial reinforcement has been shown to lead to stronger memory traces than continuous reinforcement (Humphreys, 1939; Skinner, 1938), we used a reinforcement rate of 80% for the odor pairings (based on Menz et al., 2013), which means that the CS+ sounds were presented eight times with the aversive odor and twice with the odorless vehicle. The CS- sounds were only paired with the odorless vehicle and never with the aversive odor. At the end of the emotional learning task, subjects were explicitly instructed that no odor will follow during the next run to avoid relearning, and they indicated again the valence and the arousal for the different sounds (second arousal/valence rating). Two of the four negatively learned and two of the four neutral sounds were presented again during subsequent REM or N2 sleep, depending on the sleep replay group. Sounds were 6 s long and not embedded in white noise. They were randomly presented with a randomized ISI between 7 and 13 s during sleep. The number of presentations during sleep ranged from 33 to 134 in the REM replay group and from 57 to 145 in the N2 replay group (no difference in the mean number of presentations: $t(28) = 1.12$, $p > 0.20$, see Table 1).

The recall was on the evening of day three, after a recovery night. The task was the same as during conditioning and the sounds were identical, except that no odor followed after the sounds. Since participants gain insight in the absence of odors during recall after one session, we designed the replay factor (REM sleep vs. N2 sleep) as between-subject factor containing two groups. Sounds were presented ten times, and in addition to the odor expectancy rating, participants had to indicate again the valence and arousal of the sounds during the first (third arousal/valence rating) and the last (fourth arousal/valence rating) trial.

Odor delivery and substance. We used an unpleasant odor (as confirmed by subjective odor ratings) in order to induce aversive conditioning (4-methyl pentanoic acid, 5%; Sigma–Aldrich, Germany, tested in pilot studies) and an odorless vehicle as control odor (1,2-propanediol; Sigma Aldrich, Germany). The odor and the vehicle were delivered via a 12-channel computer-controlled olfactometer designed after Lorig (2000). Room air was filtered before entering the system, and airflow was held constant at 3 l/min. To avoid tactile or thermal shifts associated with odor onset, half of the air stream was presented continuously to the subject, and only the other half was switched between room air and vehicle or odor presentation by computer-controlled valves. The olfactometer was placed in a separate room (adjacent to the subject's learning room) and was connected to the subject's mask via Teflon tubes, which allowed regulating the odor stimulation without disturbing the subject. The

subject received the odor via a small nasal mask, which assured constant stimulation but permitted normal breathing.

Cortisol, general reaction times and accuracy. To ensure that participants did not differ in their general state of wakefulness and vigilance, or in stress, they underwent a reaction time (RT) and response accuracy test and gave saliva samples before going to sleep the first time, after waking up in the morning, and before and after emotional learning and recall.

Cortisol samples were collected using Salivette collection tubes (Sarstedt, Germany) and stored at -20°C until analysis conducted by the Laboratory of Clinical Psychology at the University of Zurich. Changes in Cortisol were calculated using the trapezoid formula (Pruessner, Kirschbaum, Meinlschmid, & Hellhammer, 2003).

RTs were assessed by pressing a key as fast as possible whenever a big red disk appeared on a computer screen (as described in Little, Johnson, Minichiello, Weingartner, & Sunderland, 1998). In 40 trials, the subjects fixed their gaze on a cross, displayed for 500–1000 ms on a white screen. In 35 trials a red disk appeared, while in five random no-go trials, the screen remained white. Response accuracy was calculated as the possibility to inhibit the key press to the five no-go trials.

Skin conductance response. Skin conductance responses (SCRs) were recorded with Brain Vision Recorder (Munich, Germany). Data was collected from the non-dominant hand with Ag/AgCl electrodes filled with electrodermal electrode paste and sampled at 250 Hz. Before analysis, data was preprocessed with a customized MATLAB-based (MATLAB R2011a) script using high pass and low pass filters of 0.5 and 1.5 Hz respectively, applying a logarithmic transformation to control for the left-skewed distribution of SCR amplitudes, and by subtracting a baseline value of 1 s prior to stimulus onset. Minimal and maximal points of responses were automatically detected within the time frame of 0.9 to 3.5 s after stimulus onset to exclude effects of the UCS and cued breathing on SCR. Each of these minima and maxima were reviewed manually and amplitudes were calculated by subtracting the minimal from the maximal values. Finally, a range correction was performed to account for large inter-individual differences in electrodermal reactivity (Boucsein et al., 2012; Hauner et al., 2013; Lykken, Rose, Luther, & Maley, 1966; Menz et al., 2013).

Sleep and EEG recordings. Sleep was recorded by standard Polysomnography (PSG) with a

128-channel electrode cap (Geodesic, USA). Data was sampled at 500 Hz. For sleep scoring, data was reduced to six scalp electrodes (F3, F4, C3, C4, P3, and P4; according to the International 10–20 System) and filtered between 0.3 and 30 Hz (Iber, Ancoli-Israel, Chesson, & Quan, 2007). Additionally to the online identification of sleep stages, EEG was scored offline by three experienced lab members according to the AASM scoring manual (Iber et al., 2007). Sleep stages scored were wake after sleep onset (WASO), NREM sleep stages 1, 2, 3, REM sleep, and movement.

We analyzed if the sounds in the REM sleep replay group were presented during tonic or phasic REM sleep by visual scoring depending on the presence of eye movements.

For a more fine-grained exploratory analysis of the EEG data, EEG recordings were subjected to power spectral analysis. 30-s epochs of the whole second night half scored as N2 and REM sleep, respectively, were separated into segments of artifact-free EEG data including 2048 data points (around 4 s) with an overlap of 10% between segments. A Hanning window was applied on each data segment before calculating power spectra using Fast Fourier Transformation (FFT). Individual mean power was determined in the theta (4.5–8.0 Hz) band for REM sleep epochs, and in slow spindle (11.0–13.0 Hz), fast spindle (13.0–15.0 Hz), and combined spindle (11.0–15.0 Hz) bands for N2 sleep epochs. Power values were extracted from frontal, central and parietal electrode clusters (as described in the supplemental material of Cordi, Schlarb, & Rasch, 2014). In order to take individual differences into account, the data was normalized with an average power band between 0.5 and 50 Hz.

EEG arousal control during sound replay in sleep. To control for possible arousals in response to sound presentations, we compared alpha (8.0–11.0 Hz) power of the raw data during the 6 s of sound presentation (‘‘sound on-period’’) with the 6 s before the sound presentations (‘‘sound off-period’’). For this purpose, we computed power spectra using FFT over the 6-s segments of raw data. Individual mean power for all presented sounds was extracted in the alpha (8.0–11.0 Hz) band and averaged over all electrodes. Data was then normalized with a power band of 0.5 and 50 Hz.

Moreover, we visually scored the arousals in the EEG data during ten seconds after the sound onset. We used the arousal scoring rules of the American Sleep Disorders Association (Bonnet et al., 1992). According to these rules, arousals were scored when EEG data shifted for at least 3 s to a higher frequency (theta, alpha, and/or >16 Hz) in N2 sleep. During REM

sleep, these segments were only scored as arousal if there was also an increase in submental EMG.

The number of K-complexes was assessed by visual inspection of the EEG data during the 6 s of sound presentation. According to the AASM sleep scoring manual, we scored K-complexes when a negative sharp wave that was clearly distinguishable from the background EEG occurred for more than 0.5 s with maximal amplitudes in frontal derivations (Iber et al., 2007). We additionally defined a minimum amplitude criterion of 75µV.

Statistical analyses. Learning was split into two halves of five trials each and only the first trial of the recall was considered to avoid extinction effects (Menz et al., 2013). Data was analyzed with SPSS (SPSS19) using 2x2x2 repeated-measure ANOVAs with repetition in the factors "emotionality" (CS+/CS-), and "replay" (replay/no replay), and the between-subject factor "sleep group" (REM sleep/N2 sleep). Given the fact that the menstrual cycle can influence emotional processing, we repeated these analyses with including the half of the menstrual cycle (first half or second half) as a covariate. One participant had to be excluded from this ANCOVA because of missing data concerning the half of the menstrual cycle at testing. Furthermore, a differentiation score was calculated between the mean values of the CS+ and CS-. The difference of these scores at the beginning of recall and at the end of learning took individual values at the end of learning into account. If the Mauchly sphericity test reached significance, we displayed degrees of freedom and *p*-values that were Greenhouse–Geisser corrected. A p-value < 0.05 was considered significant.

4.3.4 Results

Sleep. Sleep parameters for the group that received sound replays during REM sleep (REM sleep group) did not significantly differ from sleep parameters in the stage N2 sleep group (N2 sleep group, all $p > 0.05$, see Table 1). The number of blocks containing sound presentations differed between the two groups, since the second half of the night contains continuous, long REM sleep episodes whereas N2 sleep is rather fragmented (mean values: REM sleep group: 2.25 ± 0.19, N2 sleep group: 3.21 ± 0.19; $t(28) = -3.56$, $p = 0.001$). Importantly, despite this difference, the number of replays did not differ between groups ($t(28) = 1.12$, $p > 0.20$; see Table 1).

Table 1. Sleep parameters.

		REM replay	N2 replay	*t*(28)	*p*
1st half	**WASO %**	3.42 ± 1.39	3.87 ± 1.40	-0.23	0.82
	N1 %	4.50 ± 0.59	4.66 ± 1.04	-0.14	0.89
	N2 %	54.45 ± 2.38	52.11 ± 2.19	0.72	0.48
	N3 %	29.06 ± 2.25	28.14 ± 2.10	0.30	0.77
	REM %	8.28 ± 1.17	10.63 ± 1.22	-1.39	0.18
	Movement %	0.29 ± 0.08	0.60 ± 0.21	-1.46	0.15
	Sleep time [min]	209.78 ± 8.19	209.14 ± 7.03	0.06	0.95
2nd half	**WASO %**	1.62 ± 0.90	1.06 ± 0.51	0.53	0.60
	N1 %	6.31 ± 0.73	4.47 ± 0.57	1.94	0.06
	N2 %	52.23 ± 1.94	56.40 ± 2.53	-1.32	0.20
	N3 %	8.88 ± 1.33	10.64 ± 1.74	-0.81	0.42
	REM %	30.73 ± 3.18	27.10 ± 2.27	0.91	0.37
	Movement %	0.22 ± 0.10	0.33 ± 0.13	-0.68	0.50
	Sleep time [min]	199.72 ± 8.95	209.32 ±10.81	-0.68	0.50
	Replays	92.44 ±7.83	81.29 ± 5.79	1.12	0.27

Wake after sleep onset (WASO), NREM sleep stages 1 and 2 (N1 and N2), slow-wave sleep (N3), and rapid eye movement (REM) sleep in % of total sleep time (sleep time). Data are means ± SEM. Right columns indicate *t*- and *p*-values for t-tests.

The percentage of sounds presented in the correct sleep stage was comparable for the REM and N2 replay group (mean values: REM sleep group: 99.28 ± 0.40, N2 sleep group: 98.66 ± 0.36; $t(28) = 1.15$, $p > 0.20$). The lack of 100% correct replay occurred because epochs with movements after sounds were scored as ‘‘movement’’ and were not included as correct target sleep stage. Moreover, offline scoring revealed that very few sound presentations were not placed in the target sleep stage when sleep stages transitioned from one to another.

EEG arousals during sound replay during sleep. The comparison of alpha (8.0–11–0 Hz) power during the 6 s of sound on-periods with the data of sound off-periods did not reveal a significant difference between on and off intervals ($p > 0.20$). Additionally, the visual scoring of arousals after sleep onset did not reveal any differences in arousals between groups (mean values: REM sleep group: 4.63 ± 0.64, N2 sleep group: 5.50 ± 0.84; $t(28) = -0.84$, $p > 0.40$).

During the visual arousal scoring of the N2 sleep replay group, we observed K-complexes which were mostly absent in the REM sleep group. Importantly, these K-complexes were not followed by EEG or EMG arousals. The comparison of the number of

visually identified K-complexes after sound onsets between the REM sleep and the N2 sleep group confirmed significantly more K-complexes during the 6 s of sound replay in the N2 sleep replay group (mean number of K-complexes: REM sleep group: 1.00 ± 0.29, N2 sleep group: 11.93 ± 2.08; $t(28) = -5.21$, $p < 0.001$).

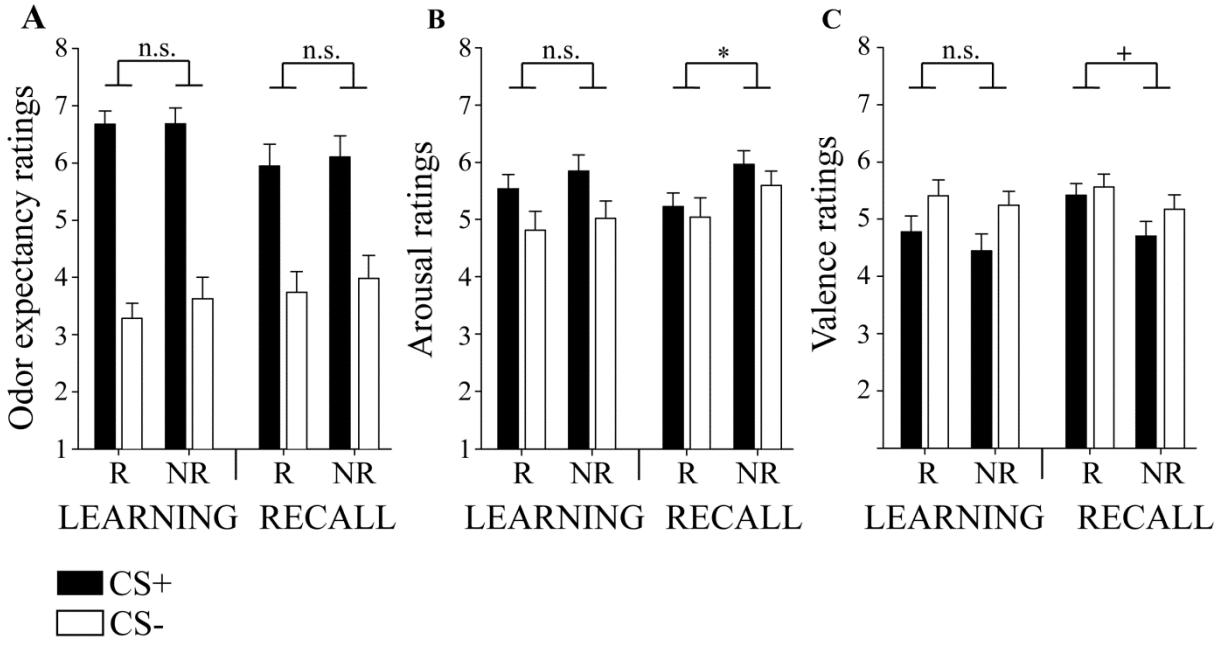

Figure 2. **A)** Odor expectancy ratings, **B)** arousal ratings, and **C)** valence ratings after learning and recall. After learning before sleep (LEARNING), expectancy ratings, arousal and valence ratings all revealed evidence for a differentiation between CS+ and CS- sounds indicative for succesful emotional learning without significant differences between later replayed (R) or non-replayed (NR) stimuli. During recall after a recovery night (RECALL), reactivation during late sleep (i.e. late REM sleep and late N2 sleep) did not influence expectancy ratings. In contrast, reactivation during late sleep significantly decreased emotional arousal ratings for both CS+ and CS- sounds, as compared to non-replayed stimuli. Similarly, a statistical trend was observed for generally increased positive valence ratings for replayed vs. non-replayed sounds. The latter effect was particularly pronounced for reactivation during REM sleep (significant interaction with sleep stage, not shown, see Table 4). Data are means ± SEM. * $p < 0.05$, + $p < 0.1$.

Learning phase

Odor expectancy. At the end of the learning procedure, the subjects successfully learned the associations between the negatively valenced CS+ sounds and the odor, as well as the safety of no odor after the CS- sounds. This was reflected in a main effect of emotion during the second half of the learning task, with a significant higher expectancy of the odor after the CS+ sounds (mean: 6.68 ± 0.20) compared to the CS- sounds (mean: 3.46 ± 0.28, $F(1,28) = 86.66$, $p < 0.001$). This ability to differentiate between the CS+ and CS- stimuli was similar for the REM sleep and the N2 sleep group ($F(1,28) = 0.08$, $p > 0.70$). There were no

differences in expectancy ratings between the stimuli we replayed during subsequent sleep ($F(1,28) = 0.79$, $p > 0.30$) and no interaction between later replay and emotionality of the stimuli ($F(1,28) = 0.40$, $p > 0.50$). Furthermore, there were no other interactions between the factors emotionality, replay, and sleep group (all $p > 0.20$; see Table 2 and Fig. 2A). No differences in reaction time of the expectancy ratings occurred between sleep groups ($F(1,28) = 0.84$, $p > 0.30$), or between emotionality ($F(1,28) = 0.26$, $p > 0.60$). Importantly, adding the half of the menstrual cycle as covariate to this ANOVA did not change the results.

Table 2. Odor expectancy ratings.

		CS+		CS-	
		R	**NR**	**R**	**NR**
Learning trials 1-5					
	REM	5.51 ± 0.22	5.78 ± 0.25	3.91 ± 0.30	4.61 ± 0.42
	N2	6.09 ± 0.23	6.14 ± 0.27	4.34 ± 0.32	4.53 ± 0.45
Learning trials 6-10					
	REM	6.62 ± 0.32	6.53 ± 0.38	3.00 ± 0.37	3.93 ± 0.51
	N2	6.74 ± 0.34	6.83 ± 0.41	3.56 ± 0.39	3.33 ± 0.55
Recall 1st trial					
	REM	6.00 ± 0.51	6.28 ± 0.51	3.88 ± 0.49	4.28 ± 0.55
	N2	5.89 ± 0.56	5.93 ± 0.54	3.61 ± 0.52	3.68 ± 0.59

Odor expectancy ratings after the first and the second half of learning, and the first recall trial. Data are means ± SEM. R: replayed, NR: non-replayed.

Arousal. Before learning, arousal ratings did not differ between sleep groups ($F(1,28) = 0.27$, $p > 0.60$). There was also no difference in arousal between later CS+ and CS- ($F(1,28) = 1.75$, $p = 0.20$) or between later replayed and non-replayed stimuli ($F(1,28) = 0.35$, $p > 0.50$). However, after learning, participants rated the CS+ as more arousing than the CS- (CS+: 5.70 ± 0.22, CS-: 4.93 ± 0.22; $F(1,28) = 6.31$, $p = 0.02$). Groups did not differ in their overall arousal ratings after learning ($F(1,28) = 0.01$, $p > 0.90$; see Table 3 and Fig. 2B). The pattern of these results was not influenced by the half of the menstrual cycle, as controlled by a repetition of this ANOVA with half of menstrual cycle as covariate.

Table 3. Arousal ratings.

		CS+		CS-	
		R	**NR**	**R**	**NR**
Before learning					
	REM	5.03 ± 0.44	5.25 ± 0.41	5.19 ± 0.39	5.31 ± 0.40
	N2	5.36 ± 0.47	5.36 ± 0.44	5.61 ± 0.42	5.93 ± 0.43
After learning					
	REM	5.34 ± 0.35	5.53 ± 0.40	4.94 ± 0.45	5.09 ± 0.41
	N2	5.75 ± 0.37	6.18 ± 0.42	4.71 ± 0.48	4.96 ± 0.44
Before Recall					
	REM	5.19 ± 0.33	6.28 ± 0.32	5.25 ± 0.46	5.59 ± 0.34
	N2	5.29 ± 0.35	5.68 ± 0.34	4.86 ± 0.49	5.61 ± 0.37

Arousal ratings before learning, after learning and before recall. Data are means ± SEM. R: replayed, NR: non-replayed.

Valence. Before learning, the two groups did not differ concerning their overall ratings of subjective valence ($F(1,28) = 0.31$, $p > 0.50$) or their valence ratings of the CS+ and CS- sounds ($F(1,28) = 3.17$, $p = 0.09$). There were also no differences in valence ratings of later CS+ and CS-, later replayed or non-replayed sounds, or interactions of these factors before learning started (all $p > 0.11$). After learning, participants rated the sounds that were previously paired with the negative odor as more negative ($F(1,28) = 5.89$, $p = 0.02$; CS+: mean: 4.61 ± 0.24, CS-: mean: 5.34 ± 0.19). Neither the valence ratings after learning between sleep groups ($F(1,28) = 0.04$, $p > 0.80$), nor other interactions or main effects between the factors differed (all $p > 0.20$; see Table 4). However, when controlling for menstrual cycle, the trend in the first valence rating before learning was no longer observed ($F(1,27) = 2.10$, $p = 0.16$). No other valence rating results were affected by the covariate.

SCRs. In contrast to the evidence for successful emotional learning with regard to expectancy ratings as well as arousal and valence ratings, we did not find differential responses for CS+ and CS- between 0.9 and 3.5 s after sound onsets at the end of the learning phase for skin conductance response ($F(1,28) = 0.12$, $p > 0.70$). There was also no difference in psychophysiological reactivity in the second half of learning between the two sleep groups ($F(1,28) = 0.61$, $p > 0.40$). Adding the half of the menstrual cycle as covariate did not impact these results.

Table 4. Valence ratings.

		CS+		CS-	
		R	**NR**	**R**	**NR**
Before learning					
	REM	3.84 ± 0.34	4.46 ± 0.35	5.00 ± 0.33	4.71 ± 0.33
	N2	4.42 ± 0.36	4.79 ± 0.38	4.75 ± 0.35	4.86 ± 0.36
After learning					
	REM	4.72 ± 0.38	4.44 ± 0.41	5.69 ± 0.38	5.06 ± 0.33
	N2	4.86 ± 0.41	4.43 ± 0.44	5.29 ± 0.41	5.46 ± 0.35
Before Recall					
	REM	5.38 ± 0.29	4.34 ± 0.37	5.94 ± 0.31	4.94 ± 0.34
	N2	5.50 ± 0.31	5.04 ± 0.40	5.21 ± 0.33	5.43 ± 0.36

Valence ratings before learning, after learning and before recall. Data are means ± SEM. R: replayed, NR: non-replayed.

Recall phase

Odor expectancy. After recovery sleep, participants still remembered which sounds were followed by the odor and which not (mean CS+: 6.03 ± 0.26; mean CS-: 3.86 ± 0.32), resulting in a main effect of emotionality for the first trial of the recall ($F(1,28) = 34.39$, $p < 0.001$). In spite of this general emotional memory effect, we did not find any influence of REM or N2 sleep replay on the expectancy ratings during the recall phase: The difference between expectancy ratings for CS+ and CS- sounds did not differ between replayed stimuli (mean: 2.21 ± 0.50) as compared to non-replayed stimuli (mean: 2.13 ± 0.54), nor between the interaction between the emotionality of the CS and replay ($F(1,28) = 0.012$, $p > 0.90$; see Table 2 and Fig. 2A). In addition, there was also no main effect of re-presentation on expectancy ratings for the emotionality of CS+ and CS- sounds ($F(1,28) = 0.40$, $p > 0.50$) and the REM and N2 sleep group did not differ ($F(1,28) = 0.55$, $p > 0.40$). These results were not influenced by the half of the menstrual cycle, as controlled by adding this as a covariate.

For a better control of inter-individual differences during the emotional learning task, we subtracted the differentiation scores for replayed and non-replayed stimuli during the second half of learning from the first recall trial. The two groups did not differ in this new score ($F(1,28) = 0.001$, $p > 0.90$), and there was also no general replay effect ($F(1,28) = 0.16$, $p > 0.60$) and no interaction between replay and sleep group ($F(1,28) = 0.91$, $p > 0.30$; see Fig. 3A). Analyses of reaction times participants needed to rate the expectancy did also not reveal any significant differences (all $p > 0.14$).

Arousal. In contrast to the results for the expectancy ratings, we indeed observed an effect of replay during late sleep on subjective arousal ratings of the sounds after the recovery night. However, and again in contrast to our hypothesis, re-presentation of the sounds during both REM and N2 sleep induced a reduction of arousal ratings for both CS+ and CS- sounds independent of emotional attribution (negative vs. neutral) (replayed sounds: 5.15 ± 0.21, non-replayed sounds: 5.79 ± 0.20; $F(1,28) = 5.92$, $p = 0.02$). This unspecific reduction in arousal for negative and neutral sounds after replay was similar for both REM and N2 sleep groups (interaction replay and group: $F(1,28) = 0.08$, $p > 0.70$). Apart from the CS-unspecific influence of replay on all sounds, we did not observe differences in arousal ratings during the recall phase between emotional valence of the CS+ and CS- sounds ($F(1,28) = 1.15$, $p > 0.20$), and no interaction between the emotional valence and the factor replay ($F(1,28) = 0.18$, $p > 0.60$; see Table 3 and Fig. 2B). Importantly, the main effect of arousal was still significant when controlling for the half of the menstrual cycle ($F(1,27) = 5.67$, $p = 0.03$), and also the pattern of the other results remained the same.

We repeated this 2x2x2 ANOVA with the arousal rating values resulting from the difference between the ratings at the end of learning and at the beginning of the recall in order to take into account non-significant baseline differences at the end of learning. The main effect of reactivation failed to reach significance ($F(1,28) = 2.07$, $p = 0.162$). When controlling for the half of the menstrual cycle, the main effect of reactivation was close to a statistical trend ($F(1,27) = 2.68$, $p = 0.11$).

In order to find out if this sleep stage-independent decrease in arousal after sound replay is predicted by any sleep parameters, we analyzed the correlations of the arousal values for the replayed sounds and the following candidate sleep parameters: sleep scorings for percentage and minutes spent in REM and N2 sleep, theta power (4.5–8.0 Hz) during the 30-s sleep epochs scored as REM sleep for both groups, and spindle (1.0–15.0 Hz), slow spindle (11.0–13.0 Hz) and fast spindle (13.0–15.0 Hz) power during the 30-s epochs scored as N2 sleep for both groups. Additionally, we correlated the number of sound replays during sleep and, for the REM sleep group only, the number of sounds replayed during tonic or phasic REM sleep, respectively. None of the sleep parameters revealed a significant correlation with the arousal ratings for the replayed sounds (all $p > 0.18$). The number of replays did not predict the reduction in arousal ($p > 0.50$), and neither did the number of replays in tonic or phasic REM sleep (both $p > 0.40$).

Interestingly, when analyzing the difference between the two differentiation scores at the end of learning (second arousal rating) and at the first trial of recall (third arousal rating)

using a 2x2 ANOVA with the factory replay (replayed/non-replayed) and sleep group (REM sleep/N2 sleep), a marginal significant interaction of replay and sleep group was revealed ($F(1,28) = 3.46$, $p = 0.07$).

The mean values showed that the ability to differentiate between CS+ and CS- was higher at recall for non-replayed sounds but declined for the replayed sounds in the REM sleep replay group (mean: replayed: -0.47 ± 0.40, non-replayed: 0.25 ± 0.38). Thus, sound re-presentation during REM sleep reduced the discrimination ability for emotional arousal for CS+ and CS- sounds from the learning to the recall phase. In contrast for the N2 sleep group, differentiation generally declined from the learning to the retrieval phase, (mean: replayed: -0.60 ± 0.43, non-replayed: -1.14 ± 0.41). Neither the factor replay nor sleep group alone reached significance (all $p > 0.10$, see Fig. 3B).

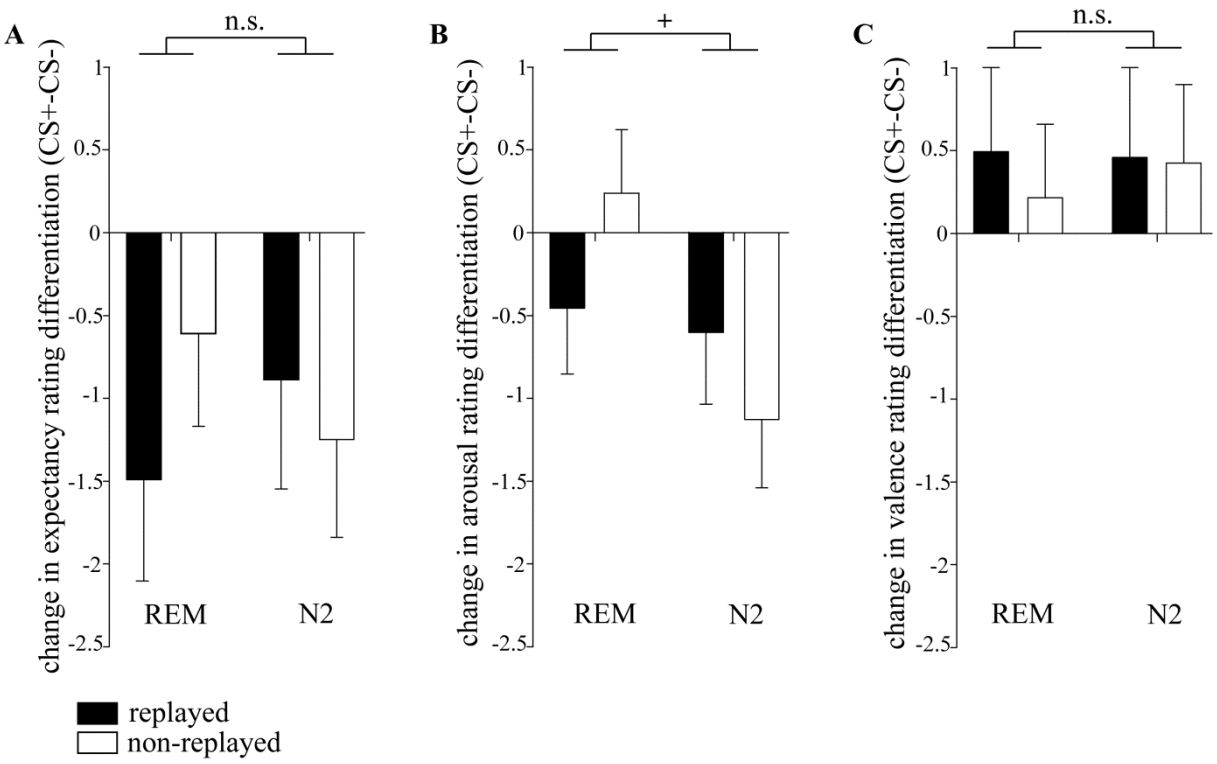

Figure 3: Change of differentiation from the end of learning to the first recall trial for **A)** odor expectancy ratings, **B)** arousal ratings and **C)** valence ratings. For all three parameters, reactivation during late sleep (i.e. late REM and late N2 sleep) did not significantly influence the ability to differentiate between CS+ and CS- sounds and therefore did not affect changes in emotional memory strength. The differentiation score was calculated by the subtracting the difference of the CS+ and the CS- during the first recall trial from the difference of the CS+ and the CS- after learning. Data are means ± SEM. + $p < 0.1$.

Valence. The CS-unspecific effect of replay on subjective arousal ratings reported above was also marginally observed for valence ratings ($F(1,28) = 2.94$, $p = 0.098$, see Fig. 3C). None of the other main effects or interactions reached significance (all $p > 0.10$; see Table 4). However, the marginal significance was no longer observed when adding the half of the menstrual cycle as covariate to the ANOVA ($F(1,27) = 2.42$, $p = 0.13$).

A differentiation score of the CS+–CS- between recall and learning did not reveal any significance (all $p > 0.30$; see Fig. 3C).

SCRs. The two groups did not differ in their SCRs between 0.9 and 3.5s after sound onsets during the first trial of recall ($F(1,28) = 0.46$, $p > 0.50$). There was also no difference in the factor replay ($F(1,28) = 1.79$, $p = 0.19$) or emotionality ($F(1,28) = 0.001$, $p > 0.90$), nor in any other interactions of these three factors (all $p > 0.30$). Including the half of the menstrual cycle did not impact these results.

Cortisol, general RT and Vigilance. The two groups did not differ concerning their saliva cortisol concentrations ($t(1,28) = -0,99$, $p > 0.30$). In addition, there was also no difference in RTs ($F(1,28) = 0.10$, $p > 0.70$) and response accuracy in inhibition ($F(1,28) = 0.87$, $p > 0.30$) between the two groups during the experiment.

4.3.5 Discussion

Our results show that replay of conditioned stimuli during late REM and N2 sleep CS-unspecifically reduced subjective arousal for both neutral and negative sounds, in contrast to non-replayed stimuli. These effects show a trend to be pronounced more in the REM replay group. However, these effects were rather weak, particularly when baseline-adjusting for arousal ratings during pre-sleep learning. Importantly, correlational analyses in this group did not reveal a differential impact of tonic or phasic REM sleep. However, neither expectancy of the CS+, nor physiological reactivity after conditioning are altered by re-presentation of emotional memory stimuli during late REM or N2 sleep.

These results are surprising and not in line with our hypotheses. Based on previous studies and theoretical accounts, we expected an enhancement of emotional memory after sound replays during REM sleep (Baran et al., 2012; Menz et al., 2013; Nishida et al., 2009; Payne et al., 2012). In particular, animal studies using Pavlovian conditioning with electrical shocks as UCS and mild electrical shocks as CS found superior emotional memory after REM

sleep re-exposure to the CS+ (Hars et al., 1985), and internal neural reactivations of emotional memory during REM sleep (possibly also involving the amygdala) have been proposed as a plausible mechanism for emotional reprocessing during this sleep stage (Maquet et al., 1996; Walker & van der Helm, 2009). However, here we show that our attempt to externally induce reactivations during REM sleep by cueing in humans has no effect on emotional memory strength. Our results are generally consistent with a recent study also showing no effects of re-exposure to emotional memories (i.e. sound-face pairs) during REM sleep (Sterpenich et al., 2014). A possible explanation for the absence of behavioral effects in humans after auditory CS+ presentations in REM sleep might be that the processing of sounds is diminished during REM sleep. However, in other studies, the presentation of previously learned information in REM sleep led to wake-like brain responses, showing that these memory traces can indeed be accessed during REM sleep (Atienza & Cantero, 2001). In fact, processing auditory stimuli appears even to be more reliable in REM sleep compared with NREM sleep, at least as indicated by ERP responses to semantic meaningful material (Bastuji, Perrin, & Garcia-Larrea, 2002). Based on this evidence, we cannot confirm a functional role of possible covert emotional memory reactivations during REM sleep since we were not able to externally bias these reactivations with auditory reminder cues, as it was done for declarative memories during SWS (Antony, Gobel, O'Hare, Reber, & Paller, 2012; Rudoy, Voss, Westerberg, & Paller, 2009).

In contrast to the lack of effects on memory strength, we observed a general reduction in emotional arousal and trend for a more positive valence after replay for both CS+ and CS- sounds, suggesting a possible role of replay during late sleep in the affective evaluation of memories. Even though the effects in arousal were slightly more pronounced during REM sleep, they were also present during late stage N2 sleep. These results are partly in line with the theoretical account that reprocessing of emotional memories during REM sleep lead to a reduction of the emotional tonus of memories (Walker & van der Helm, 2009). Our results suggest that sleep in general, and especially REM sleep, helps to decrease the experienced arousal or affective tonus, probably due to an overall integration of newly acquired memories during sleep into existing networks (Dumay & Gaskell, 2007; Ellenbogen, Hu, Payne, Titone, &Walker, 2007; Rasch & Born, 2013) and, as a result, higher familiarity with and habituation to these stimuli (Ditye, Javadi, Carbon, & Walsh, 2013). However, this result should be treated with caution, since a wake control group would be needed to definitely state that sleep in general decreases arousal and increases valence and to exclude general effects of time.

Contrary to re-exposure during REM sleep, some studies have shown effects of externally reactivating emotional memories during SWS, revealing rather inconsistent results. In rodents, cueing during SWS either impaired (Hars et al., 1985) or strengthened (Rolls et al., 2013) emotional memory using conditioning paradigms. Similarly in humans, reactivation during SWS either strengthened emotional memories, as indicated by increased reaction times, (Cairney et al., 2014) or enhanced their extinction (Hauner et al., 2013). Thus, reactivation during SWS appears to play an important role for emotional memory reprocessing, although the exact direction and mechanisms are far from clear. In our study, we excluded potential influences of spontaneous reactivation during SWS on emotional memory by specifically examining targeted reactivation during late sleep in a split-half paradigm. Future studies are needed to specifically compare effects of targeted memory reactivation on emotional memory between SWS and REM sleep using the same learning paradigm.

A clear limitation of our study is the absence of a wake group. Since we were mainly interested in the differential effect of REM compared with other sleep stages on emotional re-processing during sleep, we decided to collect data for a REM sleep and an N2 sleep control group. However, to eventually state a decisive role of sleep in general in an arousal decrease for replayed sounds, a wake group would be needed.

Furthermore, we were not able to find learning effects in the skin conductance response (SCR). A possible reason for the weak physiological reactivity during learning is that in contrast to other studies, the conditioning procedure occurred at 2 a.m. at night. In addition, we used an unpleasant odor as unconditioned stimulus. Unpleasant odors induce disgust instead of fear and their timing is less precise and controllable due to breathing in comparison with shocks or unpleasant noise (Hars et al., 1985; Menz et al., 2013; Rolls et al., 2013). Finally, retesting was done after a longer time interval of 24 h involving a recovery night. Thus, future studies should possibly test the effects of emotional memory re-exposure during sleep immediately in the morning (although see Menz et al., 2013). We additionally suggest for future studies that apply aversive conditioning to use stronger and better controllable stimuli like shocks or sounds to reliably examine effects of targeted memory reactivation on SCR.

As suggestions for similar future studies, we underline the importance of the menstrual cycle in female participants, since trends in valence ratings were no longer observed after adding the half of the menstrual cycle as covariate. Thus, possible future studies using female participants should control for the menstrual cycle. Furthermore, we used only female participants because of their better olfactory performance (Covington, Geisler, Polich, &

Murphy, 1999), and their ability to perceive the emotionality, and especially disgust, in response to odors in a more extreme way than men (Ferdenzi et al., 2013; Thuerauf et al., 2009). For more generalizable results, future studies should also include men.

In sum, here we show that replay of previously conditioned stimuli during late sleep does not affect memory strength, but influences the affective tone of both neutral and emotional stimuli. Future studies are needed to further investigate the notion that spontaneous memory reprocessing during REM sleep alters the affective tone of memories, but play no functional role for emotional memory strength.

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4.4 Study 4: Reactivation of exposure therapy success increases spindle power during NREM sleep

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4.4.1 Abstract

Exposure therapy induces extinction learning and is an effective treatment for specific phobias. Sleeping after learning promotes extinction memory and improves therapeutic success. As sleep-dependent, memory-enhancing effects are based on memory reactivation during sleep, we sought to further improve the beneficial effect of sleep on therapy success by inducing the reactivation of memories of subjective therapy success during non-rapid eye movement sleep after *in vivo* exposure-based, group therapy for the treatment of spider phobia. In addition, we investigated the oscillatory correlates of successful reactivation during sleep (i.e., sleep spindles and slow oscillations). After exposure therapy, spider-phobic patients verbalized their subjectively experienced therapy success in the presence of a contextual odor. Then, the patients napped for 90 minutes, which was recorded by polysomnography. Half of the sleep group was presented with the odor during sleep, while the other half was presented with an odorless vehicle as a control. A third group served as a wake control group without odor presentation. The reactivation of subjective exposure therapy success by odor re-exposure during sleep increased the left-lateralized frontal slow spindle (11.0-13.0Hz) and right-lateralized parietal fast spindle (13.0-15.0Hz) activity, which suggests a successful reactivation of therapy-related memories during sleep. Nevertheless, as exposure therapy was highly effective in reducing spider phobic symptoms, the effectiveness of therapy was not improved by reactivation during sleep due to a ceiling effect. Future studies should examine the possibility of enhancing therapy success by targeted memory reactivation during sleep.

4.4.2 Introduction

Specific phobias are a form of anxiety disorder that have an estimated lifetime prevalence of 11.3% among Americans (Magee, Eaton, Wittchen, McGonagle, & Kessler, 1996). To date, exposure therapy is the most effective treatment for anxiety disorders (Chambless & Ollendick, 2001), particularly for the fear of spiders (Öst, Ferebee, & Furmark, 1997). In this type of therapy, patients acquire a corrective experience in the phobic situation, which creates a new memory trace that is associated with fear extinction (Bouton, Westbrook, Corcoran, & Maren, 2006). The consolidation of this newly learned extinction memory and its integration into existing knowledge determines the reduction of symptoms. This reduction is not always guaranteed, as some patients do not respond to the treatment, while in others, only partial remission or relapse is observed (Mystkowski, Craske, & Echiverri, 2002; Mystkowski, Craske, Echiverri, & Labus, 2006).

Sleep is a promising candidate for the enhancement of treatment outcomes because of its well-known beneficial role in memory consolidation (Rasch & Born, 2013). Recognizing the high potential for sleep to consolidate a newly acquired extinction memory in a clinical setting, previous studies have examined the role of sleep in fear extinction. *In sensu* exposure of spider phobic patients by video or through virtual reality treatment resulted in an increase in post-sleep fear consolidation and generalization (Pace-Schott, Verga, Bennett, & Spencer, 2012) and to a higher reduction of subjective fear during the approach of a spider (Kleim et al., 2013). The decrease of the fear of spiders was correlated with the percentage of sleep spent in non-rapid eye movement (NREM) sleep stage two (Kleim et al., 2013). Additionally, NREM sleep has been shown to have an active role in fear extinction in healthy participants (Hauner, Howard, Zelano, & Gottfried, 2013).

Neurally, spontaneous reactivations of previously learned, hippocampus-dependent information during NREM sleep are suggested to be the mechanism by which sleep enhances memory (Wilson & McNaughton, 1994). These spontaneous memory reactivations have been successfully biased in humans. For example, by associating a visuo-spatial memory task with an odor and then re-presenting the same odor during slow-wave sleep (SWS) results in a better post-sleep memory performance compared with the presentation of an odorless vehicle (Rasch, Büchel, Gais, & Born, 2007; Rihm, Diekelmann, Born, & Rasch, 2014). Furthermore, targeted memory reactivation (TMR) with the same odor during SWS results in congruent-odor-specific changes in EEG activity, including increases in the frontal delta and parietal fast spindle activity compared with another, novel odor or an odorless vehicle (Rihm et al., 2014). However, it is still unknown whether TMR during sleep can be applied in a clinical setting to

reactivate extinction memory, as indicated by oscillatory changes during sleep, and thereby enhance therapy success.

To test this question, spider phobic patients underwent *in vivo* exposure-based, group therapy and verbalized their subjective therapy success under the presence of a background odor. Thereafter, patients stayed awake or napped with the re-presentation of the odor or of an odorless vehicle. Extinction memory was tested by changes in the subjective fear of spiders. We hypothesized that odor reactivation during sleep will result in increased delta and spindle EEG activity compared with odorless vehicle presentation. Additionally, we expected that re-presentation of the odor during sleep will result in more pronounced extinction memory consolidation, as indicated by reduced phobia-related fear compared with an odorless vehicle or an awakened patient who did not receive intervention.

4.4.3 Methods

Participants. Sixty medication-free, spider-phobic volunteers participated in this study. The diagnosis of spider phobia was based on the DSM-IV (American Psychiatric Association, 2000) and assessed by the DIA-X screening questionnaires (Essau, Wittchen, & Pfister, 1999; Wittchen & Pfister, 1997). The exclusion criteria included a diagnosis of general anxiety disorder, depression, mental disorders (with the exception of a specific phobia), sleep-related disorders, medication intake during one month prior to the experiment, major sleep disturbances or sleep rhythm changes (night shifts, shift working, time zone changes) during the eight weeks prior to the experiment, previous experience with exposure therapy, fear of moths, poor sleeping quality, and non-normal olfactory functions. Each exclusion criterion was determined and tested individually. General anxiety disorder and depression were excluded via DIA-X screening questionnaires. Additionally, the likelihood of depression, as measured by the Beck's depression inventory (Beck, Ward, Mendelson, Mock, & Erbaugh, 1961), did not differ between groups ($F(2,51) = 0.74$, $p > 0.40$). Poor sleeping quality was assessed by the Pittsburgh Sleep Quality Index (PSQI; Buysse, Reynolds, Monk, Berman, & Kupfer, 1989). Groups did not differ in PSQI values ($F(2,51) = 2.33$, $p > 0.14$). Normal olfactory function was ensured by excluding any nasal infections during the day of the experiment and by testing general olfactory performance using the "Sniffin' Sticks" inventory (Burghart, Germany). One person of the odor reactivation group had to be excluded from this general olfactory performance analysis due to missing data. The general ability to distinguish between twelve odors (mean: 11.49 ± 0.12) was comparable between groups ($F(2,50) = 0.86$,

$p > 0.40$). Additionally, subjective ratings of the general olfactory ability, which ranged from one (very poor) to five (very good), were also similar between groups ($F(2,51) = 0.03$, $p > 0.90$). All other exclusion criteria were assessed by self-report.

On experimental days, participants were instructed to end their sleep at 7.00 a.m., not to take any naps, nor to ingest alcohol- or caffeine-containing drinks.

Written informed consent was obtained after explaining the procedure. The study was approved by the ethics committee of the Faculty of Arts of the University of Zurich and in accordance with the principles of the Declaration of Helsinki (Rickham, 1964). After the first therapy session, patients were randomly assigned to three groups, which differed regarding the activity after the first therapy session.

Six participants had to be excluded from the final sample. Four participants showed a decrease in the behavioral approach test (BAT) of more than three standard deviations between two subsequent measured time points. Because of baseline differences between the three groups in the behavioral approach test, we excluded two more participants to match the baselines. Furthermore, one participant was transferred from the sleep group to the wake group because her sleep time in N2 sleep was less than five minutes, which made it impossible to present olfactory stimuli.

Thus, our final sample consisted of 54 patients (48 female, six male; mean age: 25.67 ± 6.80 years (SD); range: 18-45) in the three groups: "wake without reactivation group" (N = 18), "sleep with odor reactivation group" (N = 18), and "sleep with odorless vehicle group" (N = 18).

Design and procedure. This study consisted of two main phases: (i) pretreatment screening via e-mail and phone and (ii) treatment with two exposure-based group therapy sessions that were separated by one week. Before and after the two therapy sessions and after sleep or wake interventions, we assessed the fear of spiders by subjective arousal ratings and skin conductance responses (SCRs) during a picture task that included spiders and moths, the distance at which patients could approach a living spider, questionnaires, and saliva cortisol levels (Fig. 1).

The exposure therapy consisted of two sessions that were separated by one week and were conducted in groups of three to four patients by a trained psychotherapist. The exposure-based group therapy manual was adapted from the treatment manual that was published by Soravia et al. (2014). The first hour of both therapy sessions involved psychoeducation about spider phobia and its treatment with exposure therapy, fear circuits, avoidance behavior, and

group rules. After a five-minute break, subjects were exposed to a real spider for one hour. The exposures during the first session included looking at the spider in a wine glass and touching the spider in the glass with a pen. In the second therapy session, patients touched the spider in the glass with a finger, caught the spider with a glass, and allowed the spider to walk over their hand. Patients experienced each exposure under the direct guidance of a psychotherapist.

Both sessions occurred between 2 p.m. and 4 p.m. and concluded with a positive feedback round in which every patient reported both their personal success and experienced self-efficacy during the therapy session. After the first session, subjectively experienced therapy success was associated with an odor. Then, polysomnography (PSG) was applied to patients in the sleep group, who then slept for 90 minutes. Patients in the wake group remained awake during this time. During stable NREM sleep stages two and three, half of the sleep group was re-presented the odor of the feedback round, while the other half was presented the odorless vehicle. Awake patients were free to pursue activities of their choice, but they were instructed not to take any naps or long breaks. This was controlled by interviews and self-reports after they returned to the lab. Three months after the second appointment, we sent questionnaires to assess the level of spider phobia to all patients to measure the possible time-dependent effects of the intervention interval after the first therapy session. Patients returned these questionnaires on a voluntary basis.

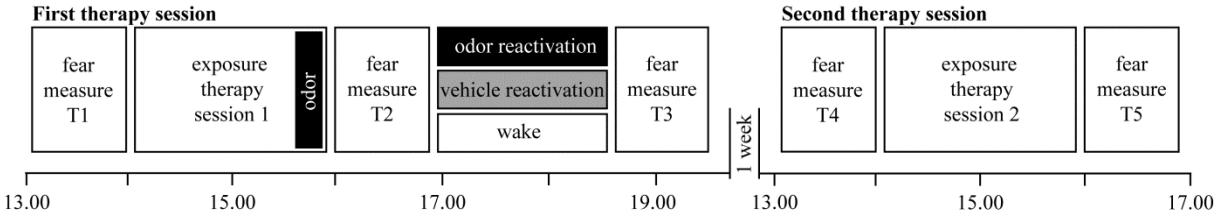

Figure 1. Procedure. Patients came to two therapy sessions separated by one week. At the end of the first therapy session, verbalized subjective therapy success was associated with an odor. Thereafter, patients slept or stayed awake. For the sleep groups, we presented the odor or an odorless vehicle during NREM sleep. Fear was measured at five different time points: As baseline before the first exposure therapy (T1), after the first exposure therapy session (T2), after the sleep or wake interval (T3), before the second exposure therapy (T4), and after it (T5).

Odor delivery and substance. The odor (magnolia and cherry blossom fragrance; Air Wick, Slough, UK) was presented during the positive feedback round at the end of the first therapy

session with the goal of associating the odor with feelings of therapy success and self-efficacy. The odor was sprayed into the room before the positive feedback round and participants inhaled it during the evaluation of their achievements during therapy. We chose a commercially available air freshener as an odor distributor because we wanted to ensure that all participants smelled the odor at the same time, which was not realizable with our olfactometer.

The odor was also diluted in odorless mineral oil (1:300; 1,2-propanediol; Sigma-Aldrich, Munich, Germany) to enable us to deliver it via an olfactometer. Because the odor was not used in previous studies, we conducted pilot studies to ensure that the odor was clearly perceivable, but not pungent at the used dilution. It was presented again for 30 s during sleep to the odor reactivation group. The odorless vehicle served as a control stimulus in the vehicle reactivation group. Thirty seconds of odor on-periods for the odor and vehicle presentation groups were alternated with 30 seconds of odor off-periods, during which the room air was presented. The odor and the vehicle were delivered via a 12-channel computer-controlled olfactometer that was designed after Lorig (2000). Room air was filtered before entering the system, and the airflow was held constant at 3 liters per minute. To avoid tactile or thermal shifts that are associated with odor onset, half of the air stream was presented continuously to the subjects and the other half was switched between room air and vehicle or odor air by computer-controlled valves. The olfactometer was placed in a separate room (adjacent to the subject's sleeping room) and was connected to the subject's mask via Teflon tubes, which allowed for the regulation of odor stimulation without disturbing the subject. The subject received the odor via a small nasal mask, which ensured constant stimulation but permitted normal breathing.

Sleep and EEG recordings. Sleep was recorded using a standard PSG with a 128-channel electrode cap (Geodesic, USA). The data were sampled at 500Hz. For sleep scoring, the EEG data were reduced to six scalp electrodes (F3, F4, C3, C4, P3, and P4; according to the International 10–20 System), re-referenced to contralateral mastoid electrodes, and filtered between 0.3 and 30 Hz (Iber, Ancoli-Israel, Chesson, & Quan, 2007). In addition to the online identification of sleep stages, EEG was scored offline by two experienced lab members according to the American Association of Sleep Medicine scoring manual (Iber et al., 2007). The sleep stages that were scored included the awake after sleep onset (WASO), NREM sleep stages N1, N2, N3, REM sleep, and movement.

For a fine-grained exploratory analysis of the effects of odor cueing during sleep, the EEG recordings were subjected to spectral analyses, spindle analysis and arousal control analyses.

Spectral power analysis. The data from the 30 s on- and off-periods of odor and vehicle stimulation were each separated into three blocks of artifact-free EEG, including 10 s of data with an overlap of 10% between blocks. A Hanning window was applied to each data block before calculating the power spectra using a Fast Fourier Transformation (FFT) with a resolution of 0.2Hz. The individual mean power was determined in the frontal slow delta (0.5-1.5Hz), frontal delta (1.5-4.5Hz), frontal slow spindle (11.0-13.0Hz) and parietal fast spindle (13.0-15.0Hz) EEG bands. The power values were extracted from the left and right frontal and parietal electrode clusters (as described in the supplemental material of Cordi, Schlarb, & Rasch, 2014). To consider individual differences, the data were first normalized with an average power band between 0.5 and 50 Hz. Then, the blocks of odor on- and off-periods were used to calculate the percent change of spectral power such that the power during the first 10-s interval of the odor on-period was expressed as a percentage of the power during the last 10-s interval of the preceding odor off-period (set to 100%). Possible differences between hemispheres were considered by analyzing the left and right hemisphere separately. Four participants in the sleep groups were excluded from the power analysis because they had fragmented NREM sleep during their nap, resulting in only one or less reactivations. Nevertheless, their total sleep time and their N2 sleep time were too long (>10 minutes) to transfer them *post hoc* to the awake group. This resulted in 32 participants for the EEG analysis (N = 16 in the sleep with odor reactivation group; N = 16 in the sleep with vehicle reactivation group).

Spindle analysis. Discrete slow (11.0–13.0Hz) and fast (13.0–15.0Hz) spindles were identified and averaged over left and right frontal (F3 and F4) and parietal (P3 and P4) EEG recording sites (Gais, Mölle, Helms, & Born, 2002; Schimicek, Zeitlhofer, Anderer, & Saletu, 1994). In brief, the power was extracted from the frequency bands of interest (11.0–13.0Hz; 13.0–15.0Hz), and the events for which the power signal exceeded a fixed threshold (±10μV) for 0.5–3s were counted as spindles. The movement of the artifact-free 10-s segments after and before odor presentation was analyzed separately in each channel (maximal difference in EMG activity of <200 μV). To calculate the spindle density, the spindle counts were divided by the number of analyzed 10-s epochs. Two participants (one in the sleep with odor

reactivation, one in the odorless vehicle presentation group) had to be excluded from this analysis because the EMG electrodes became detached during the night and the EMG criterion was not applicable. We excluded three more participants (two in the sleep with odor reactivation, one in the odorless vehicle presentation group) from the spindle analysis over parietal electrodes because they had no spindles in the analyzed off-segments. Therefore, a calculation of the count and density values by division of the on-values by the off-values was not possible.

EEG arousal count. To control for possible arousals in response to odor presentations, we visually scored the arousals in the EEG data during ten seconds after the odor onset. We used the arousal scoring rules of the American Sleep Disorders Association (Bonnet et al., 1992). According to these rules, arousals were scored when EEG data shifted for at least 3s to a higher frequency (theta, alpha, and/or >16 Hz) in NREM sleep.

Assessment of fear of spiders. Fear of spiders was measured at five different time points: before (T1) and after (T2) the first therapy, after sleep or wakefulness (T3), and before (T4) and after (T5) the second therapy. Fear measures included subjective arousal ratings, SCRs in a computer-based picture task with spider pictures, the distance at which patients could approach a living spider, subjective fear during the approach, and questionnaires about the fear of spiders. Additionally, saliva cortisol, general reaction times (RTs) and response accuracy were assessed at each time point.

Picture task. In the computer-based picture task, participants rated their arousal to 15 pictures of spiders and 15 pictures of moths, which served as neutral control pictures (Hauner, Mineka, Voss, & Paller, 2012). Additionally, their SCRs to the pictures were recorded. To avoid habituation, one unknown set of five different sets of moth and spider pictures were created, which contained pictures that were matched with regard to the size and color of the animals and background color intensity. The sets were presented in a pseudo-randomized order, whereas the 30 pictures in the set were presented randomly. After the presentation of a cross hair for 1 s, pictures of each set were presented for 7 s with a randomized ISI of 7-8s. Participants were instructed to look at the pictures for as long as possible, but if the picture became unbearable to them, they could press a key to make it disappear. After watching a picture, participants rated their arousal on a scale from one (very calm) to seven (very aroused). The difference between arousal to spider and moth pictures and the ability of

participants to look at the spider pictures for the entirety of 7 s without skipping them were used in determining the fear measurements.

BAT and VAS. After completing the picture task, the behavioral approach test (BAT) was conducted. During this task, participants approached a living spider in a closed box until the arousal was as high as possible, but still bearable. Thus, the BAT score expresses the avoidance level towards the feared object.

Participants were accompanied to the room in which the spider was placed and were allowed to approach the spider slowly and at their own speed. When participants could not approach any further, the distance to the box that contained the spider was measured. If participants were unable to enter the room because their fear was too strong, the distance to the door (600 cm) was measured as the distance value.

After the BAT, the subjectively experienced anxiety during approaching the spider was rated on a visual analogue scale (VAS) using questions about the intensity of the momentary fear, the momentary feeling of physical tension, the need to leave the situation and the most catastrophic spider-related thought, as indicated by a marking of a cross along the 10-cm horizontal line of the scale. One participant of the odorless vehicle reactivation group was excluded from the analyses of the VAS data because of missing baseline data.

SPQ and FSQ. Spider phobia at the five different time points was also assessed by the German versions of the Spider Phobia Questionnaire (SPQ; Watts & Sharrock, 1984) and the Fear of Spiders Questionnaire (FSQ; Szymanski, 1995). These two questionnaires were also sent to participants three months after the second session of the exposure therapy to assess possible time dependent changes in spider-related fear. One subject of the wake group was excluded from the analyses due to missing baseline data, resulting in 53 subjects for the SPQ and FSQ values. During this follow up, 30 of these 53 participants returned the questionnaires (reactivation with odor: N = 11, reactivation with odorless vehicle: N = 8, awake: N = 11).

SCRs. The SCRs were recorded with the Brain Vision Recorder (Munich, Germany). The data were collected from the non-dominant hand with Ag/AgCl electrodes that were filled with electrodermal electrode paste. The data were sampled at 250 Hz. To avoid movement artifacts, participants were instructed not to move during the task and the dominant hand was placed on the key with which the picture could be skipped. Before analysis, visually inspected, artifact-free data were preprocessed with MATLAB (MATLAB R2011a) by using

high pass and low pass filters of 0.5 and 1.5Hz, respectively. We then applied a logarithmic transformation to control for the left-skewed distribution of the SCR amplitudes and subtracted a baseline of 1 s prior to stimulus onset. Minimal and maximal points of responses were automatically detected during the presentation of the pictures, i.e., within the time frame of 1-7s after stimulus onset. The amplitudes were calculated by subtracting the minimal from the maximal values. Finally, a range correction was performed to account for large, inter-individual differences in electrodermal reactivity (Boucsein et al., 2012; Lykken, Rose, Luther, & Maley, 1966). Pictures that were skipped by participants after their onset were treated equally to the other stimuli if no artifacts were scored by visual inspection. This SCR data were also considered between 1 s and 7 s after the initial display of a picture. This was necessary because of the different picture presentation lengths, which depended on the time point at which participants skipped the pictures. A uniform analysis was not possible for the picture presentation times alone, so we excluded these trials.

Three participants were excluded from the analysis (reactivation with odorless vehicle: N = 2, awake: N = 1) due to missing skin conductance data at T3.

Cortisol, general reaction times and accuracy. To ensure that participants did not differ in their general state of wakefulness, vigilance, or stress, they underwent a reaction time (RT) and response accuracy test and gave saliva samples at all five fear measured time points, T1-T5.

Cortisol samples were collected using Salivette collection tubes (Sarstedt, Germany) and stored at -20°C until analysis by the biochemical laboratory at the Institute of Psychology of the University of Zurich.

RTs were assessed by pressing a key as fast as possible whenever a large, red disk appeared on a computer screen (Little, Johnson, Minichiello, Weingartner, & Sunderland, 1998). In 40 trials, the subjects fixed their gazes on a cross, which was displayed for 500–1000ms on a white screen. In 35 trials, a red disk appeared while the screen remained white in five random no-go trials. The response accuracy was calculated as the possibility to inhibit the key press during the five no-go trials.

Statistical analysis. The EEG data were analyzed by repeated measures ANOVAs with repetition in the factor “lateralization” (left versus right hemispheres) between sleep groups (odor reactivation versus odorless vehicle presentation). Significant interactions were further analyzed by post hoc t-tests between the two groups.

We conducted behavioral data analyses as performed in Kleim et al. (2013). We focused on the differences between groups between T2 and T3 (immediately after the first therapy and after the sleep or wake interval) and between T3 and T4 (after the sleep or wake interval at the end of the first session and before the second session one week later) separately by calculating the absolute and relative values. Therefore, we subtracted data at T2 from T3 and at T3 from T4. For relative changes, the data of T3 were divided by T1 and multiplied by 100. Similarly for the second session, for relative values data of T4 were divided by T3 and multiplied by 100. Calculation of the relative changes was not possible for the BAT distance scores, as most patients reached a distance of 0 cm at T3. These absolute and relative values were compared by one-way ANOVAs between the three groups.

To show the general impact of extinction learning on the fear of spiders, we focused only on the therapy sessions and analyzed the data by using a 2x2x3 ANOVA with the repeated measures factors session (first versus second session) and time (pre versus post therapy) between the groups (odor reactivation during sleep versus vehicle reactivation during sleep versus wake).

Additionally, chi square tests were conducted to analyze if the groups differed in skipping spider pictures at T3 and at T4.

A p-value of less than 0.05, as calculated using two-tailed ANOVA, was considered significant.

4.4.4 Results

Sleep and reactivation of therapy success

Sleep architecture. The total sleep time, percentage of WASO, N1, N2, N3, and movement; nor the number of olfactory stimuli presentations differed between the two sleep groups (all $p > 0.14$; Table 1). All of the odor re-presentations during sleep were placed in sleep stages N2 or N3 (100% correct placement for every patient in both groups). Only 10 of the 36 patients reached REM sleep (odor reactivation: $N = 4$; vehicle presentation: $N = 6$). Their percentage of REM sleep also did not differ ($\chi^2(1,9) = 10.00$, $p > 0.30$).

Effects of cued odor presentation on oscillatory activity. In line with our hypothesis, the EEG activity in the spindle bands during the first ten seconds of TMR in NREM sleep that was induced by the presentation of the same odor at the end of extinction learning differed from the EEG activity that was produced during the presentation of an odorless vehicle.

Over the right parietal hemisphere, the interactions between group and laterality was highly significant ($F(1,30) = 10.93$, $p = 0.002$). Relative changes in fast spindle activity (13.0-15.0Hz) from the odor off- to odor on-periods were higher in the reactivation group (106.86 ± 6.05%) compared with the vehicle group (89.27 ± 6.05%, $t(30) = 2.06$, $p = 0.049$). No difference occurred in the left parietal hemisphere (odor presentation: 100.35 ± 5.84% versus vehicle presentation: 91.02 ± 5.84%; $t(30) = 1.13$, $p > 0.2$; Fig. 2).

Table 1. Sleep parameters.

Sleep Parameters	Reactivation with odor ($N = 18$)	Reactivation with odorless vehicle ($N = 18$)	$t(34)$	p
WASO (%)	11.41 ± 2.55	18.92 ± 4.37	1.49	0.15
N1 (%)	15.26 ± 1.76	12.59 ± 1.72	-1.08	0.29
N2 (%)	47.26 ± 4.70	41.52 ± 3.60	-0.97	0.34
N3 (%)	24.50 ± 5.66	23.22 ± 4.34	-0.18	0.86
REM (%)[a]	4.27 ± 0.66	8.97 ± 2.18		0.35
Movement (%)	0.63 ± 0.23	0.72 ± 0.25	0.24	0.81
Total time (min)	92.61 ± 7.03	84.50 ± 7.24	-0.80	0.43
Number stimulations	28.56 ± 3.69	35.56 ± 4.36	-1.23	0.23

Wake after sleep onset (WASO), NREM sleep stages 1 (N1) and 2 (N2), slow-wave sleep (N3), and rapid eye movement (REM) sleep in % of total sleep time (sleep time). Right columns indicate *t*- and *p*-values for t-tests. Data are means ± *SEM*.

[a]Only 10 out of the 36 participants reached REM sleep (reactivation with odor: $N = 4$; reactivation with odorless vehicle: $N = 6$; df =9; χ^2 test).

Interestingly, a reversed lateralization pattern was observed in the slow spindle band (11.0 – 13 Hz). Again, the interaction between the reactivation group and laterality was significant ($F(1,30) = 6.65$, $p = 0.02$). Patients with odor reactivation during sleep revealed higher changes in slow spindle activity over the left frontal hemisphere (115.18 ± 6.80%) compared with the odorless vehicle group (94.87 ± 6.80%, $t(30) = 2.11$, $p = 0.04$). No difference occurred over the right frontal hemisphere (odor presentation: 104.05 ± 5.71% versus vehicle presentation: 99.25 ± 5.71%, $t(30) = 0.59$, $p > 0.50$; Fig. 2).

The finding of increased spindle activity following odor re-presentation was paralleled in the distinct sleep spindles. Namely, we observed changes in the fast parietal spindle counts that were distinctly enhanced in the odor reactivation compared with the odorless vehicle

group, although no effect of lateralization was observed (odor presentation: 172.84 ± 24.54% versus vehicle presentation: 95.33 ± 25.47%; $F(1,25) = 4.80$, $p = 0.04$).

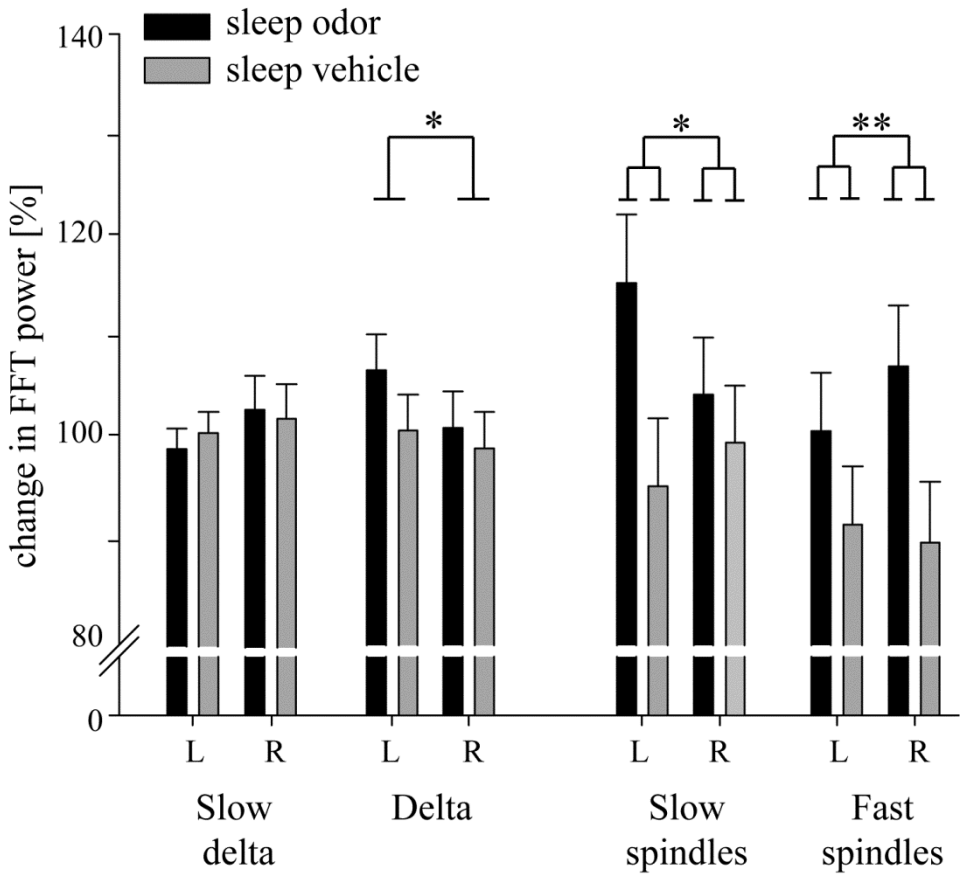

Figure 2. Changes in relative EEG activity during the first 10s of odor-on intervals compared with the last 10s of odor-off intervals. Data for slow delta (0.5–1.5Hz), delta (1.5–4.5Hz) and slow spindle (11.0-13-0Hz) activity are retrieved from frontal electrodes. Data for fast spindle activity (13.0–15.0 Hz) are retrieved from parietal electrodes. L: left hemisphere, R: right hemisphere. Data are means ± SEM. *p* values from one-way ANOVAs are indicated (*$p < .05$, **$p < .01$).

A similar pattern was observed for changes in the frontal slow spindle counts (135.93 ± 8.44% vs. 98.67 ± 8.44 %; $F(1,28) = 9.74$, $p = 0.004$). Changes in the spindle density were also increased following odor presentation compared with odorless vehicle presentation for frontal slow spindles (odor presentation: 108.74 ± 5.27%, odorless vehicle presentation: 91.66 ± 5.27%; $F(1,28) = 5.25$, $p = 0.03$) and demonstrated a statistical trend for parietal fast spindles (odor reactivation: 142.40 ± 21.18 %, odorless vehicle presentation: 88.31 ± 21.98 %; $F(1,25) = 3.14$, $p = 0.09$). In these analyses, neither a lateralization main effect nor a lateralization* condition interaction was found.

Contrary to the two spindle bands, we observed no effect of reactivation in frontal delta activity, neither as an interaction with laterality nor as a main effect of the group (both $p > 0.20$; Fig. 2). Independent of the group, the frontal delta activity (1.5-4.5Hz) in the left hemisphere (103.46 ± 2.53) was significantly higher than in the right hemisphere (99.67 ± 2.60; $F(1,30) = 4.95$, $p = 0.03$). This decrease was greater than in the reactivation group (Fig. 2). The reactivation had no impact on the frontal slow delta power (0.5-1.5Hz, all $p > 0.06$). Importantly, odor presentation did not result in changes in the EEG arousals after odor onset compared with the odorless vehicle presentation ($t(30) = 0$, $p = 1.0$; odor reactivation: mean = 1.38 ± 0.30; vehicle presentation: mean = 1.38 ± 0.30).

Fear of spiders. After showing that odor presentation during sleep successfully reactivated exposure-related memory traces, as indicated by changes in the oscillatory spindle activity, we tested whether reactivation of subjective therapy success during sleep affected the fear of spiders.

In general, exposure therapy was highly efficient and significantly reduced the fear of spiders, as measured by approach behavior (BAT), subjective fear ratings (VAS), spider phobia questionnaires (SPQ, FSQ), electrodermal activity (SCRs), and subjective arousal during exposure to spider pictures. In particular, we found a main effect of time (all $p < 0.001$) and session (all $p < 0.001$) as well as a significant time by session interaction effect (all $p \leq 0.02$; except for FSQ: $p = 0.08$) for all of these measures. These effects indicated a decrease in fear after the therapy sessions compared with before the sessions and a decrease in fear from the first to the second session. The interaction reflected a stronger reduction of fear during the first than during the second session.

Despite the great efficacy of our therapy in generally reducing symptoms in the three groups, we observed no evidence that reactivation during sleep or sleep in general improved therapy success compared with the wake control group.

BAT and VAS. The ability to approach a living spider was not influenced by sleep or reactivation during sleep compared to the wake group ($F(2,51) = 0.48$, $p > 0.60$), and differences between the intervention groups were also not observed one week later ($F(2,51) = 0.26$, $p > 0.70$; Fig. 3a). There were also no differences between groups when analyzing the differential changes between each of the succeeding pairs of measured time points (all $p > 0.50$; Table 2).

Absolute and relative VAS ratings at all four scales also did not differ between the three groups at T3 (all $p > 0.20$) and at T4 one week later (all $p > 0.40$; Fig. 3b).

Picture task. Similarly, the difference in subjective arousal towards phobic and non-phobic pictures was the same for the three groups at T3 ($F(2,51) = 0.42$, $p > 0.60$) and one week later at T4 ($F(2,51) = 2.21$, $p = 0.12$). The groups also did not differ in their duration of looking at spider pictures at T3 ($\chi^2(2,52) = 1.04$, $p > 0.50$) or at T4 ($\chi^2(2,52) = 1.66$, $p > 0.40$; Fig. 3c).

SPQ and FSQ. None of the questionnaires revealed differences between the three groups at T3 (all $p > 0.15$) or at T4 (all $p > 0.70$) in absolute or relative values (Figs. 3d and 3e).

Three months after therapy, the three groups still did not differ in their absolute or relative values in the FSQ and SPQ scores compared with the baseline before the first (all $p > 0.17$) and after the second therapy session (all $p > 0.30$).

Table 2. Baseline values and differential changes in distance in cm during the behavioral approach test (BAT).

Time point	Reactivation with odor (N = 18)	Reactivation with odorless vehicle (N = 18)	Wake (N = 18)	F (2,51)	p
T1	123.56 ± 26.80	119.17 ± 32.45	93.17 ± 18.05	0.39	0.68
T2-T1	-90.22 ± 24.13	-87.01 ± 23.74	-59.78 ± 14.84	0.62	0.54
T3-T2	-8.28 ± 8.04	-1.00 ± 6.03	-8.50 ± 3.66	0.48	0.62
T4-T3	10.28 ± 11.71	20.00 ± 12.02	20.28 ± 9.51	0.26	0.77
T5-T4	-29.83 ± 7.86	-30.94 ± 15.69	-33.44 ± 42.14	0.03	0.98
T5-T2	-27.83 ± 6.92	-11.94 ± 11.55	-21.67 ± 27.12	0.87	0.43

Data is presented each pair of succeeding time points T1 (beginning of the first session), T2 (after the first exposure therapy), T3 (after the sleep/wake interval), T4 (one week later, beginning of second session) and T5 (after the second exposure therapy) and for the decrease between the two therapy sessions. Negative values indicate a closer approach to the spider compared with the time point before. Data are means ± SEM. Right columns indicate F and p-values for one-way ANOVAs.

SCRs. At every time point T1-T4, patients revealed an increase in physiological reactivity to spiders compared with their reactivity to moth pictures, independent of the group (all $p < 0.005$); however, after the second therapy at T5, the SCRs to spiders and moths were

comparable ($F(2,48) = 1.49$, $p > 0.20$). The SCRs of three groups were comparable at all time points (all $p > 0.12$).

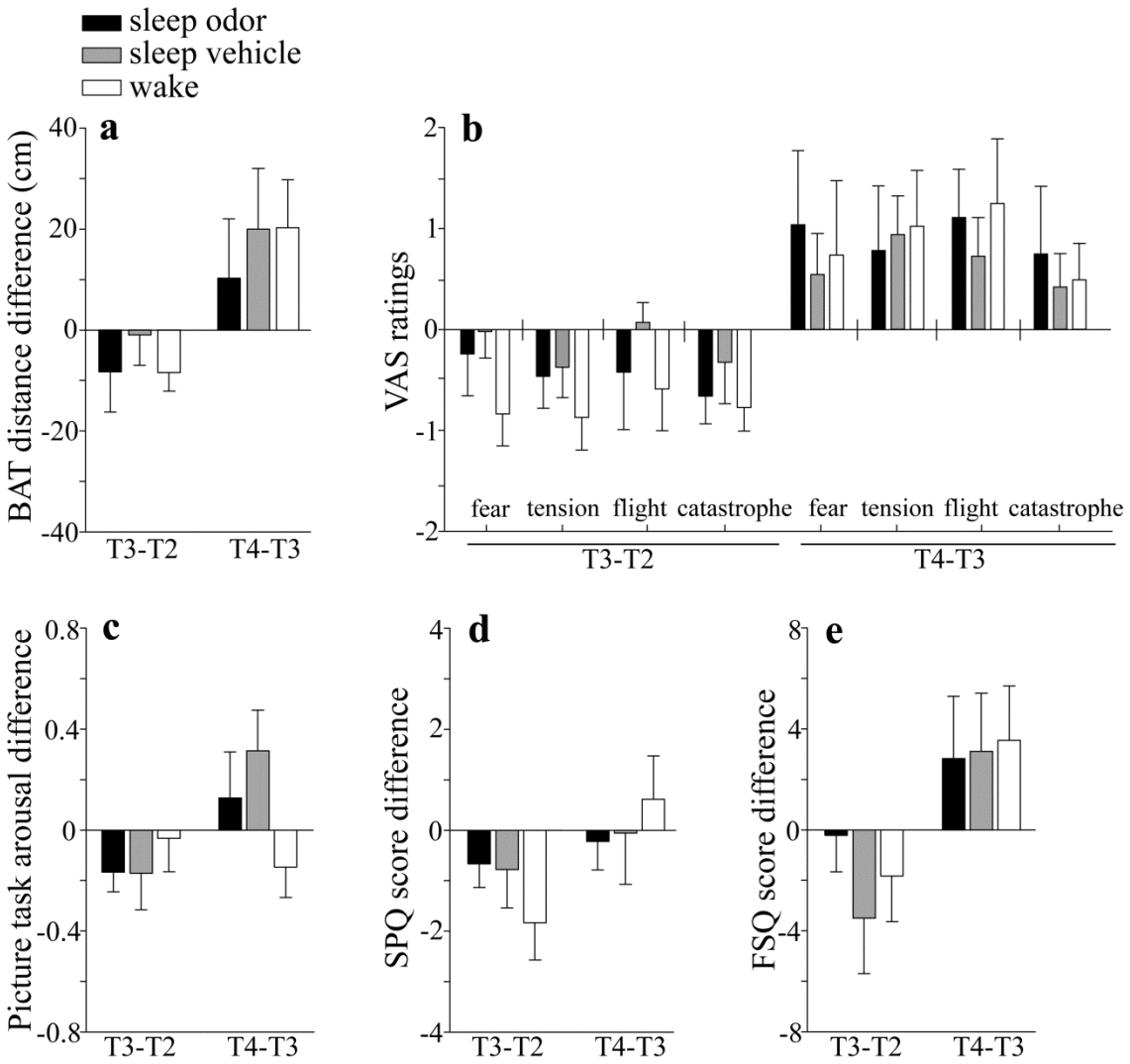

Figure 3: Absolute changes in fear parameters from time point T2 (after the first exposure therapy) to T3 (after sleep/wake) and from T3 (after sleep/wake) to T4 (one week later). Changes are presented for the following measures: (A) behavioral approach test (BAT), (B) fear during BAT measured by four different visual analogue scales (VAS) for momentary fear (fear), momentary feeling of physical tension (tension), need to leave the situation (flight) and catastrophic spider-related cognitions (catastrophe), (C) the differences in subjective arousal between moth and spider pictures, (D) scores for the spider phobia questionnaire (SPQ), and (E) scores for the fear of spiders questionnaire (FSQ). Negative values indicate a decrease in fear compared with the time point before. Data are means ± SEM. Right columns indicate *F* and *p*-values for one-way ANOVAs.

Cortisol. The three groups did not differ in their absolute or in their relative changes in the levels of cortisol at T3 (both $p > 0.20$) or at T4 (both $p > 0.40$).

General RT and accuracy. At each time point, we assessed the general RT and response accuracy in an RT test. The three groups did not differ in the general RT or in the response accuracy after sleep or during wakefulness at T3 (both $p > 0.70$) or at T4 (both $p > 0.40$).

4.4.5 Discussion

Our results show that the olfactory TMR of subjectively experienced therapy success during NREM sleep results in both increased frontal slow and parietal fast spindle activity compared to patients who experienced a presentation of an odorless vehicle. Nevertheless, contrary to our hypothesis, these changes in EEG activity were not present in the delta band and did not result in differences in fear of spiders between the groups. Thus, our findings indicate that cueing subjective therapy success by odor presentation is possible and that changes in electrophysiological spindle activity are observable. However, because *in vivo* exposure group therapy might cause a ceiling effect in extinction learning, extinction memory could not be improved by sleep or by reactivation during sleep. Alternatively, cueing of the verbalized therapy success might not have been potent enough to induce therapy success.

Previous studies support the notion of a critical role of NREM sleep in fear extinction (Hauner et al., 2013; Kleim et al., 2013). The finding that TMR with the same odor presented during learning alters EEG activity during NREM sleep agrees with this notion and with our previous findings (Rihm et al., 2014), although in the present study, this effect depended on lateralization. Our results suggest that the neural mechanisms of fear extinction consolidation during NREM sleep might be similar to those of declarative memory consolidation, where thalamo-cortical spindles during SWS sleep establish a dialogue between the hippocampus and neocortex, thereby enabling information transfer between these brain regions (Mölle & Born, 2011).

The asymmetric left lateralization for slow spindle activity and right lateralization for parietal fast spindle activity during olfactory TMR is a new finding. A possible explanation for the lateralization is the strong emotional involvement during verbalizing subjective therapy success. Increases of left-lateralized frontal EEG activity have been associated with tasks that induce a positive mood (Davidson & Fox, 1982; Ekman & Davidson, 1993) and with greater trait approach motivation (Amodio, Master, Yee, & Taylor, 2008; Coan & Allen, 2003). In our study, a positive mood and a motivation to approach the feared object were recorded following patient verbalization of subjective therapy success and associated self-

efficacy. Right posterior regions have been involved in the processing of emotional stimuli. For example, both positive and negative ERP components of emotional stimuli are larger over right parietal regions (Kayser et al., 1997; Keil et al., 2001; Thomas, Johnstone, & Gonsalvez, 2007). Importantly, we can exclude arousals or awakenings in the reactivation group as possible explanations of the observed changes in spindle spectral power.

The results of our study do not support the findings of previous studies in which sleep was shown to enhance extinction memory at a behavioral level and thus reduce fear following exposure. In contrast to our *in vivo* exposure-based group therapy, these studies used *in sensu* therapy with a virtual environment (Kleim et al., 2013) or a short movie clip (Pace-Schott et al., 2012). A reason for the missing behavioral effects of sleep on extinction memory consolidation after our *in vivo* exposure therapy could be a possible ceiling effect of extinction learning. Exposure therapy has been shown to be an effective treatment, particularly for spider phobia (Choy, Fyer, & Lipsitz, 2007; Soravia et al., 2014), even after one session (Öst, Brandberg, & Alm, 1997; Öst, Ferebee, et al., 1997; Öst, 1996). Because we applied a well-established experimental effect to a real psychotherapeutic situation in a group format, it was not possible to create a highly controlled experimental setting, as was done in studies that investigate the effect of sleep on declarative memory, where it was common to define a fixed learning criterion (e.g., 60% of the overall learning material; (Rasch et al., 2007; Rihm et al., 2014) or place a subject within a certain diameter from the target stimulus (Rudoy, Voss, Westerberg, & Paller, 2009).

Due to the absence of the immediate effects of sleep on the treatment outcome after a virtual reality treatment (Kleim et al., 2013), we re-measured fear one week after the initial therapy. Nevertheless, the different sleep groups and the wake group did not differ at this time point, nor did they differ three months later. Importantly, we can eliminate the different vigilance states between the groups, particularly after sleep intervention, as having an effect on the behavioral results because we compared the reaction time tests and correct responses in a go-no go test at every time point.

Because we applied a group therapy setting, it was not possible to individually associate the extinction learning process with the odor. Anxiety in the observing patients, in addition to the acting patient, would eventually have led to the association of fear with the odor. Moreover, after a successful odor-extinction association, the subsequent extinction trials of other patients with the presentation of the odor could have weakened the new memory trace because TMR on declarative memory during wakefulness results in the destabilization of the newly acquired memory trace (Diekelmann, Büchel, Born, & Rasch, 2011). However, we

cannot eliminate the possibility that cueing only the verbalized therapy success, which is abstract compared with specific actions during extinction learning, might have been too weak to enhance extinction memory. When considered together, re-exposure to *in vivo* exposure-based, group therapy for spider phobia during NREM sleep results in lateralized increases in slow and fast spindle bands. This impact on the EEG could be reflected by a reduced fear of spiders if exposure learning is not already at the ceiling. For future studies, we suggest using other extinction learning paradigms (e.g., *in sensu*) to further explore the present findings.

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4.5 Study 5: Sleep deprivation increases dorsal nexus connectivity to the dorsolateral prefrontal cortex in humans

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4.5.1 Abstract

In many patients with major depressive disorder, sleep deprivation, or wake therapy, induces an immediate but often transient antidepressant response. It is known from brain imaging studies that changes in anterior cingulate and dorsolateral prefrontal cortex activity correlate with a relief of depression symptoms. Recently, resting-state functional magnetic resonance imaging revealed that brain network connectivity via the dorsal nexus (DN), a cortical area in the dorsomedial prefrontal cortex, is dramatically increased in depressed patients. To investigate whether an alteration in DN connectivity could provide a biomarker of therapy response and to determine brain mechanisms of action underlying sleep deprivations antidepressant effects, we examined its influence on resting state default mode network and DN connectivity in healthy humans. Our findings show that sleep deprivation reduced functional connectivity between posterior cingulate cortex and bilateral anterior cingulate cortex (Brodmann area 32), and enhanced connectivity between DN and distinct areas in right dorsolateral prefrontal cortex (Brodmann area 10). These findings are consistent with resolution of dysfunctional brain network connectivity changes observed in depression and suggest changes in prefrontal connectivity with the DN as a brain mechanism of antidepressant therapy action.

4.5.2 Introduction

Sleep deprivation has been used for decades as a rapid-acting and effective treatment in patients with major depressive disorder (MDD) (1, 2). Although clinically well established, the mechanisms of action are largely unknown.

Brain imaging studies have shown that sleep deprivation in depressed patients is associated with renormalized metabolic activity, mainly in limbic structures including anterior cingulate (ACC) as well as dorsolateral prefrontal cortex (DLPFC) (3–6), and that changes in limbic and DLPFC activity correlated with a relief of depression symptoms (7–9). Recent studies in patients with depression point to a critical importance of altered large-scale brain network connectivity during the resting state (10, 11). Among these networks, the default mode network (DMN), which mainly comprises cortical midline structures including precuneus and medial frontal cortex as well as the inferior parietal lobule (12–15), is most consistently characterized. In functional magnetic resonance imaging (fMRI) studies, the DMN shows the strongest blood oxygenation level–dependent (BOLD) activity during rest and decreased BOLD reactivity during goal-directed task performance. The DMN is anticorrelated with the cognitive control network (CCN), a corresponding task-positive network, which encompasses bilateral fronto-cingulo-parietal structures including lateral prefrontal and superior parietal areas (16). A third system with high relevance for depression—the affective network (AN)—is based in the subgenual and pregenual parts of the ACC [Brodman area (BA) 32] (17). The AN is active during both resting and task-related emotional processing, and forms strong functional and structural connections to other limbic areas such as hypothalamus, amygdala, entorhinal cortex, and nucleus accumbens (18, 19).

Increased connectivity of DMN, CCN, and AN with a distinct area in the bilateral dorsomedial prefrontal cortex (DMPFC) was recently found in patients with depression compared with healthy controls (20). This area within the DMPFC was termed dorsal nexus (DN) and was postulated to constitute a converging node of depressive “hot wiring,” which manifests itself in symptoms of emotional, cognitive, and vegetative dysregulation. This led to the hypothesis that a modification in connectivity via the DN would be a potential target for antidepressant treatments (20).

Recent studies in healthy subjects reported reduced functional connectivity within DMN and between DMN and CCN in the morning after total (21) and in the evening after partial sleep deprivation (22). However, brain network connectivity via the DN was not examined in these studies. Given the recently proposed role of the DN in mood regulation, here we specifically tested whether sleep deprivation as a well-known antidepressant treatment modality affects connectivity via the DN. Based on our previous findings on network changes by ketamine (23), we hypothesized that sleep deprivation leads to a reduction in connectivity via the DN.

4.5.3 Methods

Study subjects. Healthy female subjects (n = 12, mean age, 23.42 ± 3.12 [SD]) without any psychiatric, neurological, or medical illness were self-referred from online study advertisements. The study was approved by the University of Zurich institutional review board, and subjects gave written informed consent before screening. All subjects underwent a psychiatric interview and medical examination. Exclusion criteria were a history of psychiatric/neurological diseases, sleep–wake cycle abnormalities, drug abuse, concurrent medication, cardiovascular disease, anemia, or thyroid disease, MR exclusion criteria, and pregnancy. The week before the experiments, subjects were obliged to follow a regular sleep–wake pattern with bedtimes between 10:00 PM and 8:00 AM. Wrist actigraphy was assessed for the sleep deprivation night and the following day.

Sleep deprivation and experimental protocol. Subjects underwent two fMRI measurements—once well-rested after normal sleep and once after sleep deprivation—at 6:00 PM or 8:30 PM (randomized, two subjects scanned after each other, scanning time kept constant). During sleep deprivation, participants slept from 3:06 AM (± 1:36 h [SD]) until 6:48 AM (± 2:48 h [SD]), with a sleep duration of 3 h 42 min (± 1:40 h [SD]), and then stayed awake until the measurement the next evening. Such an early sleep deprivation protocol may elicit an antidepressant response in distinct cases, particularly in younger women (47). The experiments were performed with an interval of two days. Participants had to abstain from caffeine and alcohol on the experimental days. Assessments were performed at the neuroimaging center of the Psychiatric Hospital of the University of Zurich and the Child and Adolescence Psychiatry, sleep deprivation was conducted at home.

EEG recording during fMRI scanning. Presence of wakefulness and sleep during scanning was confirmed by standard polysomnographic recordings using a 32-channel MR-compatible EEG montage (Brain Products). Impedances were kept below 20 kΩ. In addition to online control for sleep, the polysomnographic records were visually scored offline by two independent raters according to standard criteria (48).

To quantify the EEG during the 8-min resting-state intervals, records were subjected to power spectral analysis. Data of all channels were filtered between 0.3 and 35 Hz with a slope of 48 dB/oct and rereferenced to averaged mastoids. Artifacts were excluded. Power spectra between 0 and 25 Hz (fast Fourier transform routine; 10% Hanning window; 0.2-s overlap) of artifact-free, 4-s EEG epochs sampled with 1,024 Hz were calculated with Brain

Vision Analyzer. Individual mean power across all channels was determined for the following EEG bands: 0.5–4.5 Hz (delta), 4.5–8.0 Hz (theta), 8.0–11.0 Hz (alpha), 11.0–15 Hz (beta 1), and 15.0–25.0 Hz (beta 2).

Functional MRI data acquisition. Measurements were performed on a Philips Achieva 3.0T TX 3-tesla whole-body magnetic resonance unit equipped with an eight-channel head coil array. The subjects were told to lie still in the scanner with their eyes closed during the acquisition of resting-state data. The functional images were collected in 8-min runs (196 volumes) using a sensitivity-encoded single-shot echo-planar sequence (TE = 20 ms; field of view = 22 cm; acquisition matrix = 88 × 85, interpolated to 96 × 96, voxel size = 2.50 × 2.50 × 2.50 mm3, reconstructed to 2.29 × 2.29 × 2.5 mm3, and sensitivity-encoded acceleration factor R = 2.5) sensitive to BOLD contrast (T2* weighting). Using a midsagittal scout image, 42 contiguous axial slices were placed along the anterior–posterior commissure plane covering the entire brain with a repetition time of 2,500 ms. A 3D T1-weighted anatomical scan was obtained for structural reference.

Postprocessing and fMRI data analysis. Standard image data preparation and preprocessing, as well as statistical analysis and visualization were performed with the software BrainVoyager QX (Brain Innovation BV). Functional data preprocessing included a correction for slice scan timing acquisition, a 3D rigid body motion correction, a spatial smoothing (Gaussian kernel of 4 mm full width half maximum), a temporal high-pass filter with cutoff set to two cycles per time course, and a temporal low-pass filter (Gaussian kernel of 3 s). Structural and functional data were coregistered and spatially normalized to the Talairach standard space using a 12-parameter affine transformation. In the course of this procedure, the functional images were resampled to an isometric 3-mm grid covering the entire Talairach box. Nuisance signals (global signal, white matter, and cerebrospinal fluid signals) were regressed out from each data set together with motion translation and rotation estimates after segmenting the entire brain, the white matter, and ventricles from the normalized T1 volume.

A seed-based analysis (49, 50) was performed to study the functional connectivity from the PCC and DN to the entire brain similar to previous studies (20, 23). A control seed region was defined by tracing a 6-mm radius sphere centered at (x = −8, y = −60, z = 21). This region was anatomically located in the precuneus (51), and fell slightly more posteriorly and more inferiorly, albeit not overlapping, to the PCC seed. To compute functional

connectivity maps corresponding to a selected seed region of interest (ROI), the mean regional time course was extracted from all ROI voxels and correlated against all voxels of the brain. Two ROIs were studied, the definition of which was based on our previous work (23), and separate correlation maps were produced for each subject, condition, and ROI. The correlation maps were applied the Fisher's r-to-z transform $z = 0.5 \text{ Ln } [(1 + r)/(1 - r)]$ before entering a second-level random-effects statistical analysis where the main and differential effects of the two studied conditions were summarized as t-statistic maps. This analysis was carried out by treating the individual subject map values as random observations at each voxel, thereby one- and two-sample t tests were performed at each voxel to map the whole-brain distribution of the seed-based functional connectivity for the single condition and the difference between the two conditions. The statistical maps were thresholded at $P = 0.05$ (corrected for multiple comparisons) and overlaid on the average normalized T1 volume of all subjects. To correct for multiple comparisons in the voxel-based analysis, regional effects resulting from the voxel-based comparative tests were only accepted for compact cluster surviving the joint application of a voxel- and cluster-level statistical threshold chosen with a nonparametric randomization approach. Namely, an initial voxel-level threshold was set to $P = 0.005$ (uncorrected) and a minimum cluster size was estimated after 1,000 Monte Carlo simulations that protected against false positive clusters up to 5% (52).

4.5.4 Results

Both after normal sleep and sleep deprivation, posterior cingulate cortex (PCC) seed-based analysis revealed a clear depiction of the DMN, with positively correlated clusters in orbital cortex and DMPFC, inferior parietal lobule, and precuneus (all bilateral) and negatively correlated clusters in medial frontal and lateral prefrontal cortex (Fig. 1). The DMN was determined using a seed region identical to a previous work (center of this region: $x = -8$, $y = -49$, $z = 28$) (23). It is important to note that, consistent with previous studies (22, 24), sleep deprivation reduced functional connectivity of the PCC with the ACC (BA 32; coordinates: +5, +43, +3; z score = 3.7173, $P = 0.000201$; cluster size = 359 mm^3) (Fig. 2). On the other hand, when we selected a seed region in the precuneus, the resulting functional connectivity analysis yielded no differential effects between sleep deprivation and normal conditions over the entire brain at the used statistical threshold.

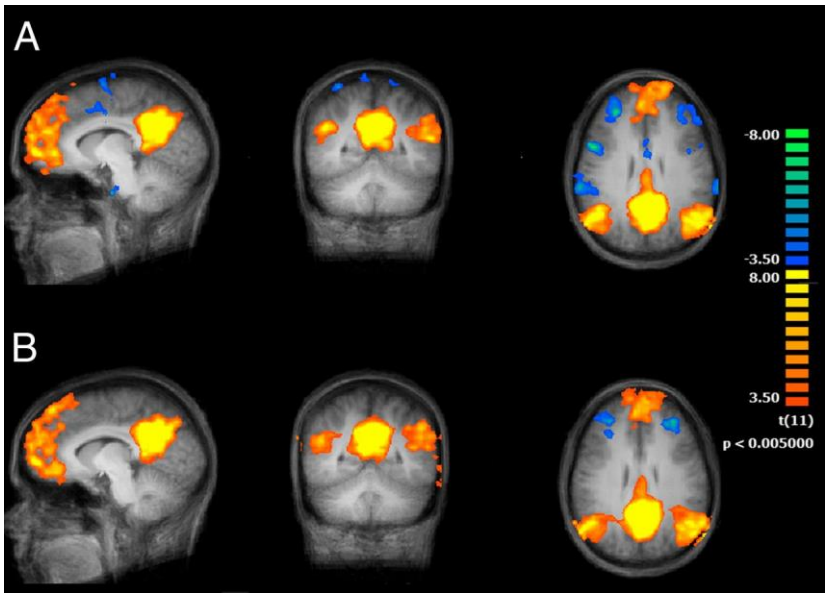

Figure 1. Functional connectivity maps (n = 12) of the brain using the PCC as seed region and comparing two conditions, normal wakefulness (A) and sleep deprived (B) (main effects, $P < 0.05$ cluster corrected).

The DN seed-based analysis also allowed a delineation of the main DMN nodes. The overlap with the DMN, however, was only partial. The contribution of positively connected regions was more prominent in the anterior part of the brain, including putative regions of other networks such as CCN and AN, and less prominent in the posterior part of the brain (Fig. 3).

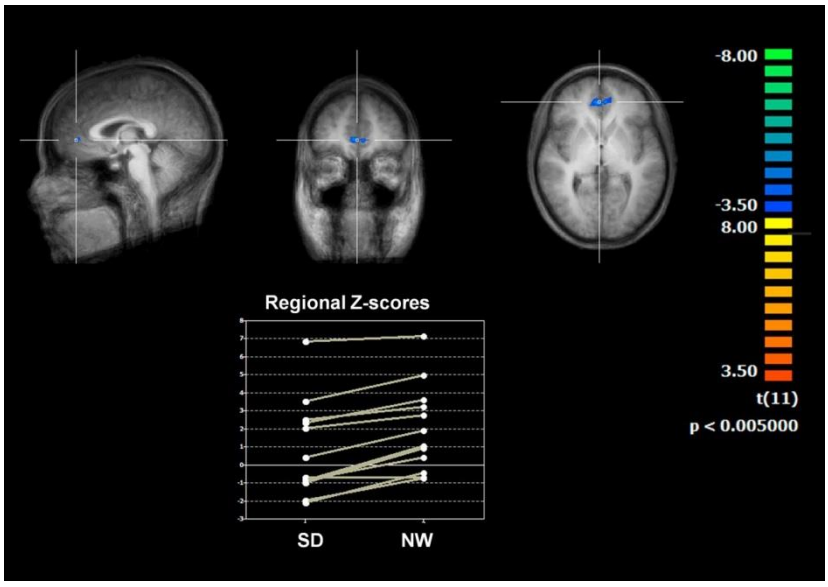

Figure 2. The contrast of the two conditions shows a reduced connectivity between the PCC seed and the bilateral ACC (BA 32; coordinates: +5, +43, +3; z score = 3.7173, significance = 0.000201; cluster size = 359 mm) after sleep deprivation (SD) compared with normal wakefulness (NW).

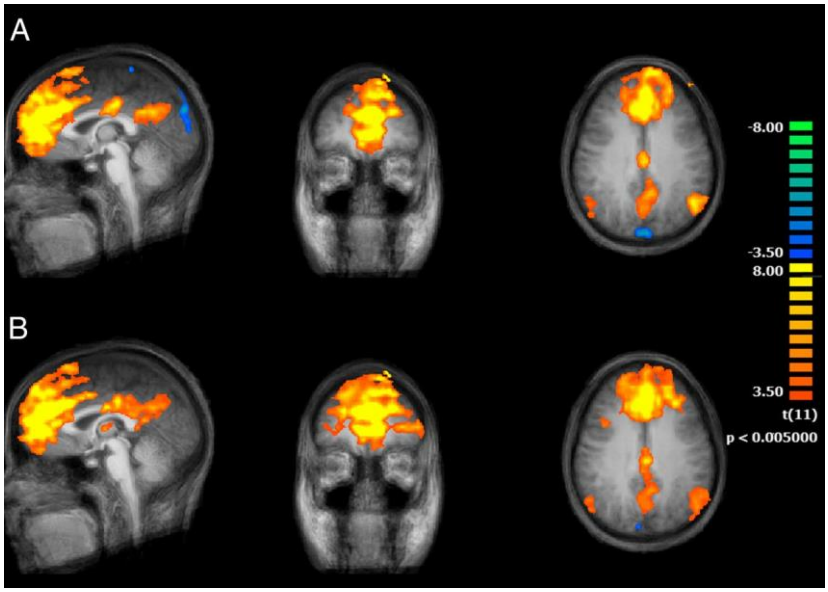

Figure 3. Functional connectivity maps (n = 12) of the brain using the DN as seed region and comparing the two conditions, normal wakefulness (A) and sleep deprived (B) (main effects, $P < 0.05$ cluster corrected).

It is interesting that, in contrast to the known effects of sleep deprivation on DMN connectivity, DN connectivity was altered in unexpected manner. More specifically, compared with the normal sleep condition, DN connectivity after sleep deprivation was significantly increased in right anterior middle (coordinates: +38, +46, +18; z score = 3.5413, significance = 0.000498; cluster size = 324 mm^3) and superior frontal gyri (coordinates: +23, +46, +18; z score = 4.202, significance = 0.000026; cluster size = 1,092 mm^3), both located in DLPFC and pertaining to the dorsolateral part of BA 10 (border to BA 46; Fig. 4). These frontal regions appear consistently and positively correlated with DN only in the sleep deprivation condition, whereas in the normal wakefulness condition, half or more of the subjects exhibited negative scores.

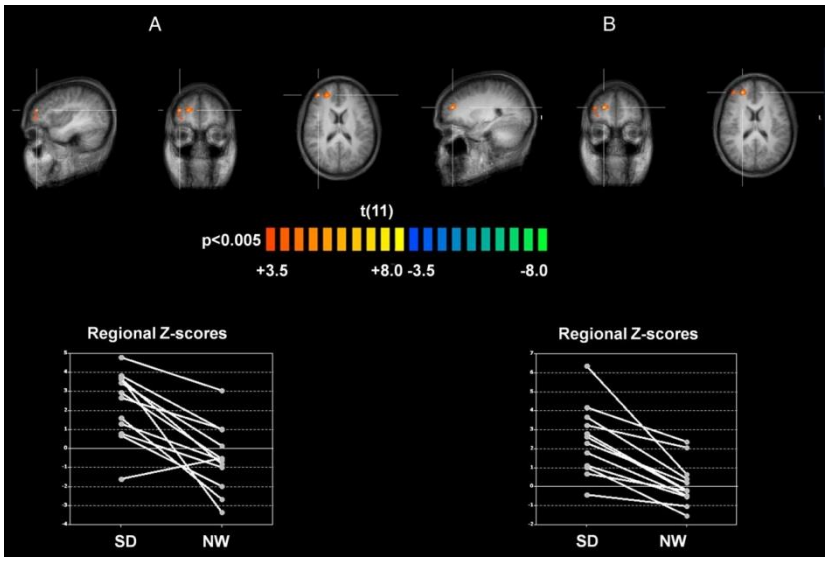

Figure 4. The contrast of the two conditions shows an increased connectivity between the DN seed and two areas (A) on the right middle (BA 10, DLPFC, spot 1: coordinates: +38, +46, +18; z score = 3.5413, significance = 0.000498; cluster size = 324 mm) and (B) on the right superior frontal gyrus (BA 10, DLPFC; spot 2: coordinates: +23, +46, +18; z score = 4.202, significance = 0.000026; cluster size = 1,092 mm) after SD compared with NW.

A comparison of the DMN connectivity changes and the DN connectivity changes due to sleep deprivation (Fig. 5) shows a reduction of the DMN–CCN anticorrelations from the normal wakefulness (Fig. 5 A and B) to the sleep deprivation (Fig. 5 C and D) condition. Although it does not reach statistical significance, which might be due to the small sample size, this tendency is in line with previous studies (21, 22). More important, this comparison clarifies that the main effect of sleep deprivation on the functional connectivity of the DN is not to reduce DMN anticorrelations in these regions, but rather to promote their positive correlation to the DN.

To control vigilance during fMRI, the EEG was recorded. In both conditions, subjects were awake for more than 95% of imaging time, and no differences in vigilance states were found between the conditions **(***P* > 0.6). As expected, spectral power in delta and theta frequencies (<8 Hz) was slightly enhanced after prolonged wakefulness, yet did not differ significantly from the normal wakefulness condition (p_{all} > 0.3; Table 1).

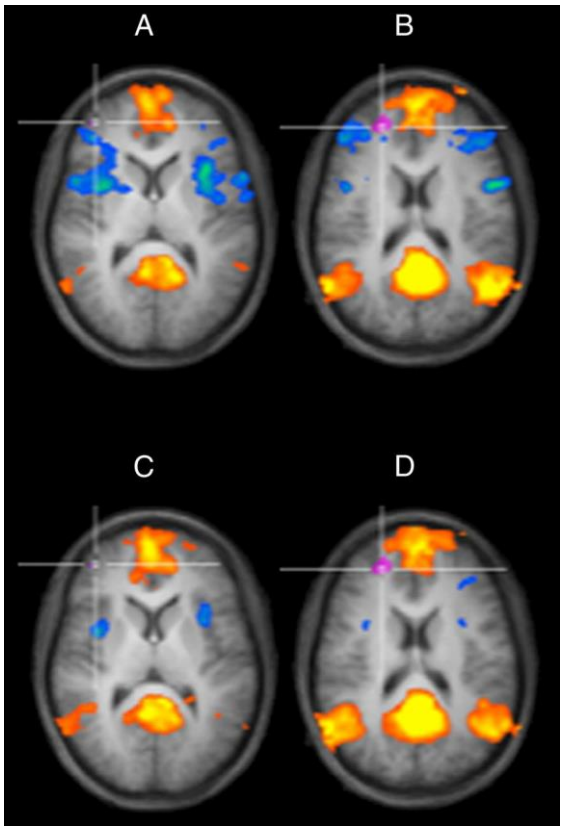

Figure 5. Comparison of the locations of the DMN-CCN spots of Fig. 1 after (A) normal wakefulness and (C) SD with the DN spots of Fig. 4 after (B) normal wakefulness and (D) SD. An axial cut was generated at the location of the DN spots, keeping the positive and negative correlations of the PCC seed overlaid on the same average anatomy to show in detail the location of these spots with respect to the CCN spots.

Table 1. Results of EEG spectral analysis during fMRI scanning

	Mean NW ± SEM	Mean SD ± SEM	Mean Diff. ± SEM	*t*-value	*P*-value
Delta (0.5-4.5 Hz)	4.64 ± 0.57	5.28 ± 0.65	0.65 ± 0.64	1.01	0.33
Theta (4.5-8.0 Hz)	4.29 ± 0.82	4.59 ± 0.78	0.30 ± 0.60	0.49	0.64
Alpha (8.0-11.0 Hz)	2.01 ± 0.42	2.06 ± 0.40	0.05 ± 0.21	0.23	0.82
Sigma (11.0-15.0 Hz)	1.17 ± 0.21	1.26 ± 0.33	0.09 ± 0.24	0.38	0.71
Beta (15.0-25.0 Hz)	0.96 ± 0.81	0.87 ± 0.56	-0.09 ±0.25	-0.35	0.74

Means ± SEM (n = 12) represent EEG spectral power values in consecutive frequency bands (delta, theta, alpha, and beta) averaged over all recording electrodes during fMRI scanning in normal wakefulness and sleep deprivation conditions.

4.5.5 Discussion

Here we delineate a differential pattern of resting-state connectivity after sleep deprivation, which is a potent, rapidly acting antidepressant intervention with a largely unknown mode of

action. More specifically, using resting-state fMRI in healthy subjects, we found that sleep deprivation reduced functional connectivity between the PCC and the bilateral ACC (BA 32), yet increased connectivity between the DN and two areas within the right DLPFC (BA 10). Although the previous observation was consistent with our a priori hypothesis, the latter observation was not expected. Visual inspection and quantification of the EEG during scanning confirmed that the changes in connectivity were not caused by the occurrence of spontaneous sleep after prolonged waking.

There is growing evidence that brain connectivity within the DMN and between the DMN and other brain networks is altered in patients with MDD compared with healthy volunteers, primarily reflecting dysfunctional self-referential processing such as rumination, negative anticipation, and excessive feelings of guilt and shame (11). We explored the functional connectivity of the PCC as a core seed of the DMN, after normal wakefulness and sleep deprivation in healthy subjects. In both conditions, we found a clear depiction of the DMN (13), including the PCC, orbital and dorsal parts of the medial prefrontal cortex (MPFC), inferior parietal lobule, and precuneus (all bilateral; Fig. 1). The comparison between normal sleep and sleep deprivation revealed a significantly reduced connectivity of the PCC to the bilateral ACC (BA 32) after sleep deprivation (Fig. 2).

The ACC is seen as a key structure for emotional processing and depressive psychopathology and is part of the AN (25). Numerous studies using event-related and resting-state fMRI designs point to alterations in ACC activity in depressed patients (26–29). The first resting-state fMRI study with MDD patients revealed a hyperconnectivity between the subgenual ACC and the DMN, confirming previous PET studies, which found resting state overactivity in ACC in these patients (30). Hyperconnectivity in the MPFC and ventral ACC was correlated with rumination in another fMRI study (31). Here, we found that sleep deprivation reduced connectivity on the ACC–PCC axis. This is of particular interest because resting-state DMN dominance is associated with increased maladaptive, depressive rumination, and reduced adaptive, reflective rumination (32). Depressed patients typically fail to down-regulate DMN activity during emotional stimulation (33, 34). Our finding of reduced intrinsic DMN connectivity after sleep deprivation resembles a pattern of normalization with regard to the depressive state. Keeping in mind the multiple functional connections of the ACC, a connectivity reduction to the DMN might be viewed as a potential therapeutic effect in patients who suffer from excessive ACC network contribution during rest, as it is observed during rumination.

Our study focused on the DN as a specific seed region, which plays a crucial role in the pathophysiology of depression, and was recently discovered as a node mediating dramatic functional hyperconnectivity between DMN, CCN, and AN in patients with MDD (20). This hot wiring via the DN was proposed to underlie core depressive symptoms such as rumination and hyperarousal, as well as affective and vegetative dysregulation. A reduction of DN connectivity may, thus, represent a neurobiological target for antidepressant treatment strategies and a potential biomarker of antidepressant response. Two recent studies tested this so-called DN hypothesis using psychopharmacological challenges in healthy subjects. The first revealed reduced functional connectivity between DN and hippocampus after 7 d of citalopram administration (35). The second showed reduced DN connectivity with the PCC and the pregenual ACC 24 h after ketamine infusions (23). These studies support the hypothesis that reducing DN connectivity to subcortical structures and the DMN network may represent a biomechanism of antidepressant action.

In the present study, we observed similar DN connectivity to the key structures of the DMN and the AN after normal sleep and sleep deprivation. We found in both conditions significant DN connectivity to the bilateral PCC, bilateral precuneus, and bilateral parietal lobules (Fig. 3), indicating that the DN is highly correlated with intrinsic DMN connectivity in healthy subjects. Compared with normal sleep, sleep deprivation induced an expansion of the DN connectivity pattern with the DMPFC toward more dorsolateral regions in the deprivation condition. This effect led to the delineation of two sleep deprivation–related areas of significant hyperconnectivity in the right anterior middle and superior frontal gyrus, both of which are located in DLPFC (BA 10/BA 46; Fig. 4). These areas showed negative scores in the normal wakefulness condition in half or more of the subjects, suggesting the possibility that these regions could be anticorrelated with the DN in their initial status. From a metabolic perspective this means that sleep deprivation may actually weaken the anticorrelation, rather than increasing the correlation of these regions to the DN. However, as a differential effect, we observe a change in the functional connectivity distribution where these regions are initially not part of a positive or negative DN network, which in turn seems to recruit them specifically in the sleep deprivation–altered condition.

The comparison of the functional connectivity changes of the DMN and DN networks after normal wakefulness and sleep deprivation (Fig. 5) shows that the negative correlations supporting the DMN–CCN anticorrelation result weakened in the sleep deprivation condition in line with previous studies. Furthermore, the DN region functionally connects to a frontally emphasized DMN due to its overlap with the DMPFC node of the PCC-based DMN pattern.

After sleep deprivation, new regions in the superior and lateral frontal cortex participate to this network, although these regions are not part of the CCN.

Our results are consistent with several PET studies highlighting the importance of two distinct brain structures for the pathophysiology and treatment of depression, namely the ACC and the DLPFC.

Baseline brain metabolism, as measured with PET, is consistently increased in MDD patients in limbic structures such as the MPFC and ACC, and is consistently reduced in the DLPFC (36–41). In depressed patients, selective serotonin reuptake inhibitor (SSRI) treatment resulted in increased metabolic activity in the middle frontal gyrus (42) and the prefrontal cortex (43). Furthermore, a PET study showed that increased ACC activity before antidepressant sleep deprivation was correlated with reduction in depression symptoms (4). It is intriguing that the positive treatment response following sleep deprivation correlated with decreased activity in the ACC (BA 32) and increased metabolism in areas including the right DLPFC (BA 46). This relationship of depression symptom relief with a metabolic decrease in inferior and orbital frontal areas and an increase in the DLPFC was recently partly confirmed with a higher resolution MRI scanner (6). In addition, patients with bipolar depression who responded to sleep deprivation had decreased activity in the ACC (BA 32, 24) and an increased activity in the DLPFC (BA 10, 46) in reaction to negative visual stimuli during an fMRI task (comparing pretreatment to posttreatment) (7). Increased prefrontal responsivity to cognitive demands after sleep deprivation was also observed in healthy subjects (44). Wu et al. and Benedetti et al. interpreted the increase of DLPFC activity—which correlated with symptom improvement—as a sleep deprivation–related reactivation of top–down control on negative emotional processing (6, 7). This interpretation is supported by reports of DLPFC reactivity in relation to voluntary suppression of sadness (45) and decrease of DLPFC metabolism in PET after induction of transient sadness in healthy subjects (46). Regarding brain network connectivity, the DLPFC is a canonical structure of the CCN, representing conscious control of executive functions and mental representations (16).

In conclusion, the current pattern of altered resting-state connectivity induced by sleep deprivation—dissociation of ACC from DMN and recruitment of CCN to DN—may indicate a shift from affective to cognitive network contributions to the DMN, which could be beneficial in depressed patients who suffer from excessive ACC and/or impaired DLPFC function. Therefore, our data warrant an extension of the DN hypothesis and the integration of differential changes in brain network connectivity into the framework of mechanisms underlying antidepressant effects of sleep deprivation. Further research is needed to identify

potential therapeutic benefits of matching altered functional connectivity patterns in depression with corresponding patterns of action of antidepressant treatments.

4.5.6 Acknowledgments

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4.5.7 Author contributions

O.G.B., P.S., F.E., B.R., and E.S. designed research; O.G.B., J.S.R., P.S., J.B., and B.R. performed research; J.S.R., M.S., H.-P.L., F.E., and B.R. analyzed data; O.G.B., H.-P.L., J.B., F.E., B.R., and E.S. wrote the paper; and E.S. provided funding.

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5 Discussion

The purpose of this section is the combined discussion of the findings of the five studies with respect to the initial hypotheses and their embedding in the existing literature. First, the study results will be interpreted with respect to sleep-dependent memory consolidation and its application to emotional memory processing and extinction memory consolidation. In the end, future directions in sleep research and suggestion for future studies will be presented.

5.1 Specifying the neural mechanisms of sleep-dependent memory consolidation

As hypothesized, the results of the first study confirm the effectiveness of TMR. Additionally, also in line with our hypotheses, we found high odor specificity for TMR. This was evidenced by the fact that the presentation of an olfactory stimulus during SWS only resulted in a memory-enhancing effect in response to the same odor. In contrast, memory performance after the presentation of a novel odor during SWS was comparable to an odorless vehicle. The presentation of reminder cues during SWS elicited changes in oscillatory events implicated in sleep-dependent memory consolidation, namely in fast spindle power, delta power, and in slow oscillation slopes, which also confirmed our assumption. Concerning the differences in olfactory processing during sleep and wakefulness, our assumption that odor processing during sleep compared with wake results in a hyporesponsiveness of the piriform cortex was also confirmed. Surprisingly, no differences were seen during olfactory processing between wakefulness and sleep for other brain regions associated with memory.

The results of study one can be interpreted in line with the assumptions of the neural mechanism behind sleep-dependent memory consolidation. It is suggested that system consolidation is facilitated by slow oscillations which particularly occur in SWS and orchestrate memory consolidation during sleep by synchronizing hippocampal sharp wave-ripples via spindles (Mölle & Born, 2011). More detailed, the up-state of the slow oscillations synchronizes these thalamo-cortical spindles and hippocampal sharp wave-ripples. Interestingly, in line with the importance of the up-states of the slow oscillations, another study found that auditory TMR during SWS only led to a memory-enhancing effect during retrieval if these tones were coupled to up-states, whereas a presentation during down-states did not affect memory (Ngo, Martinetz, Born, & Mölle, 2013). By the synchronizing influence of the up-states during sleep, a transfer between the hippocampus and the neocortex

is enabled, facilitating system consolidation (Mölle & Born, 2011). The neurobiological pathway between hippocampus and neocortex is usually inhibited during wakefulness due to high concentrations of the neurotransmitter acetylcholine, impeding a hippocampal-neocortical transfer of information. In contrast, cholinergic levels are very low during SWS (Hasselmo, 1999). Furthermore, the hippocampal sharp wave-ripple events have been associated with memory replay during both, sleep (Lee & Wilson, 2002; Nádasdy et al., 1999; Skaggs & McNaughton, 1996) and wakefulness (Csicsvari et al., 2007; Diba & Buzsáki, 2007; Foster & Wilson, 2006; Karlsson & Frank, 2009). Altogether, sleep therefore provides a perfect environment for system consolidation.

Importantly, presentation of a memory-associated cue did not only lead to increases in delta power, but also in steeper negative-to-positive slopes of slow oscillations. These slopes are associated with the transition from neural down-states to up-states and possibly reflect a more pronounced synchronization of cortical networks as a response to olfactory TMR. The steeper slopes of the slow oscillations additionally predicted memory performance and could thereby reflect a synchronizing influence of hippocampal sharp wave-ripples on slow oscillations. This suggestion further underlines a strong functional connection between the different oscillatory events.

Since the presentation of a memory-associated cue during SWS triggers hippocampal activity (Rasch et al., 2007), we can assume that our congruent odor re-presentation during SWS activated newly acquired, and hence still hippocampus-dependent memory traces. Interestingly, a result of this hippocampal replay bias was seen in slow oscillatory events. As previously described, the model of active system memory consolidation assumes a top-down influence of neocortical slow oscillations on hippocampal sharp wave-ripples via spindles (Mölle & Born, 2011). The most important conclusion of these findings is that the system can also work in the other direction, which means in a bottom-up way with hippocampal sharp wave-ripples orchestrating spindles and neocortical slow oscillations. The mechanism involved in memory consolidation can probably be triggered in a bottom-up manner, by the hippocampus. Since our speculations are based on EEG data, these results would need to be replicated in animal studies, where direct cell derivations can be recorded.

The widely accepted effectiveness of olfactory TMR on declarative memory consolidation raises the assumption that odors are processed differently during sleep and wakefulness. Especially the hyporesponsiveness of the piriform cortex after odor presentation in sleep, as shown in an animal study (Barnes et al., 2011), nourishes this assumption. Despite this hyporesponsiveness, the facts that odors are incorporated into dreams (Trotter, Dallas, &

Verdone, 1988), can influence the valence of dream reports (Schredl et al., 2009), and are able to enhance consolidation if they were previously associated with memory (Diekelmann et al., 2011; Rasch et al., 2007) show that odors are actively processed during sleep. This leads to the conclusion that other brain areas could be involved in odor processing during sleep. However, we were not able to confirm these assumptions. We indeed found hyporesponsiveness in the piriform cortex in response to olfactory stimuli during sleep compared with wakefulness. But our results did not reveal increased activation of hippocampal or amygdaloid regions during sleep compared with wakefulness.

A possible interpretation of this finding could be that a preferential access of odors to memory-associated regions during sleep is given, but not present in response to a mere olfactory stimulus alone. Rather, an association of the odor with a previously acquired memory trace is needed to find possible preferential hippocampal activation during sleep in response to odors (Rasch et al., 2007). This finding fits in line with the results of the first study, where the presentation of a novel odor that was not memory-associated did neither lead to changes in oscillatory events nor in behavioral results. Since we suggest a bottom-up influence of the hippocampus on the process of system memory consolidation, an effective, hippocampus-activating TMR needs to involve a memory-associated odor. Therefore, the suggestion for future studies is a replication of experiment two, but with a declarative memory task that includes odors as background memory cues and a presentation of these cues during SWS in the fMRI scanner as in a previous, similar study (Rasch et al., 2007). Additional analyses like fMRI pattern analyses that compare sleep and wake brain activity patterns would be of interest here. Additionally to future fMRI studies investigating the neural correlates of olfactory TMR, it would also be extremely interesting to compare memory-associated olfactory with auditory TMR with regard to effectiveness and magnitude of hippocampal activation induced by these two methods.

In sum, we could confirm the active system consolidation hypothesis with our results. Our study further evidences a synchronizing bottom-up influence of hippocampal sharp wave-ripples on neocortical slow oscillations. Importantly, the effectiveness of odors in eliciting hippocampal activity is closely bound to a former established association of this odor with memory. This is evidenced by an absence of increased hippocampal activation during mere odor processing in sleep, and by the absence of effects on oscillatory events in response to a novel odor. Further animal studies using direct cell recordings are needed to confirm a possible bottom-up influence of the hippocampus on the neocortex. Moreover, fMRI studies

where memory-associated sounds and odors are presented could investigate possible differences in neural processing of TMR stimuli of these different modalities.

The effectiveness of TMR on declarative memory consolidation and its ability to give insight in underlying neural mechanisms is widely acknowledged and can be useful in further determination of the role of sleep in the processing of other memory types.

5.2 Application of the sleep-dependent memory consolidation effect on emotional memory processing

Concerning emotional memory processing during sleep, we could not confirm the hypothesis that covert reactivations of previously learned emotional memory during REM sleep are causal for emotional memory processing during sleep. Also emotional reactivity was not selectively influenced by TMR during REM sleep.

One reason for the missing results of emotional memory TMR during REM sleep could be that different mechanisms than assumed are involved in emotional processing during sleep. The "sleep to forget and sleep to remember" model suggests covert reactivations of emotional memories during REM sleep which should lead to increased memory and less emotional reactivity at retrieval (Walker & van der Helm, 2009). In line with our non-findings concerning emotional memory consolidation, a recent study investigating the effects of REM sleep on emotional memory by re-presenting CS+ sounds during post-learning REM sleep did also not find increased emotional memory consolidation (Sterpenich et al., 2014). Based on these findings, one could argue that the perception of sound stimuli is reduced or suppressed during REM sleep, resulting in an ineffectiveness of TMR. However, studies revealed that presenting previously learned information during post-learning REM sleep elicited wake-like brain responses (Atienza & Cantero, 2001). Moreover, ERP responses to meaningful semantic material were even more pronounced during REM compared with NREM sleep (Bastuji, Perrin, & Garcia-Larrea, 2002).

In contrast to our and Sterpenich's and colleagues (2014) results, another recent study found strengthened emotional memory consolidation after TMR of emotional memory during SWS (Cairney, Durrant, Hulleman, et al., 2014). The interpretation of these differences in the results is speculative, but one reason could be the nature of the emotional learning task. The learning paradigm of Cairney and colleagues (2014) consisted of hippocampus-dependent location learning of emotional pictures and additionally contained context sounds with

congruent valence as TMR stimulus. Thus, the most part of their task is hippocampus-dependent. In fact, their operationalization of emotional learning consists in hippocampus-dependent declarative learning with a slightly negative tone (induced by negative pictures and according sounds). This can explain the effectiveness of TMR during SWS.

Furthermore, emotional pictures as negative UCS are also associated with a declarative component and the emotional pictures in this task are maybe not emotional enough to induce aversive learning. Consequently, these memories have a strong hippocampal component and profit from TMR during SWS. Of note, we did not create a hippocampus-dependent context association by using background cues in our paradigm, but we re-presented the negatively learned stimulus. Therefore, our paradigm should reflect hippocampus-independent aversive learning with a strong amygdaloid component.

In contrast, stimuli to elicit strong negative emotions by inducing for example fear are electrical shocks. As a recent study could show, post-sleep SCRs to previously learned pictures-shape associations paired with shocks were correlated positively with the amount of REM sleep (Menz et al., 2013). Unfortunately, this study did not use TMR during REM sleep. All in all, these findings evidence that the sleep stage during which emotional memory is re-processed might be critically depend on the nature of the task.

Likewise, a reason for ineffectiveness of emotional memory TMR during REM sleep could be missing UCS emotionality or a lack in detecting emotional memory changes. We used not many trials, and especially for the analysis of the recall values we only included the first trial in the analysis. Maybe a paradigm using a larger number of trials would be more sensitive in finding effects of emotional memory reactivation at recall. Moreover, our aversive olfactory stimulus, compared with very emotional stimuli like electrical shocks that elicit fear, rather induced disgust. On the other hand, we confirmed the aversion against the UCS with a pretest, and thus all participants subjectively perceived the UCS as clearly negative. Furthermore, the UCS induced emotional learning, as seen in odor expectancy ratings after learning. However, these consideration lead to the suggestion for further studies to use an electrical shock as UCS, since human studies could already show a sleep-dependent effect on emotional memory learning based on shock-associations (Menz et al., 2013).

Besides consolidation of the informational core of the emotional memory, the SFSR hypothesis also suggests reduced reactivity towards emotional stimuli. As reviewed in the theoretical background, few studies can show reduced emotional reactivity after post-learning sleep (Gujar, McDonald, et al., 2011; Rosales-Lagarde et al., 2012; Sterpenich et al., 2007; Van Der Helm & Walker, 2011), whereas the majority of studies found enhanced emotional

reactivity (Baran et al., 2012; Groch et al., 2013; Lara-Carrasco et al., 2009; Menz et al., 2013; Pace-Schott et al., 2011; Payne & Kensinger, 2011; Sterpenich et al., 2009; Wagner et al., 2002; Werner et al., 2015). In contrast to the absence of an influence of TMR on emotional memory consolidation, we found a reduction of emotional arousal after sleep, but it was a general reduction for neutral and negative stimuli, and thus not specific with regard to emotional attribution of the CS. This however, is not in line with the SFSR hypothesis, since the reduction in arousal was also given for stimuli presented during N2 sleep and that did not have a negative valence. A possible explanation for our finding is habituation to the stimuli presented during sleep which could result in a higher familiarity during retrieval and thus a reduction in arousal. A clear limitation of the study is the absence of a wake control group which could help with interpreting for example the arousal results and to provide evidence for the sleep specificity of this effect.

These above mentioned points underline the difficulty of establishing and controlling emotional learning in an experimental setting. Consequently, it would be wrong to exclude a role of sleep, and REM sleep, in emotional processing and emotional memory processing. Before drawing such causal conclusions, future studies are needed with other paradigms to investigate if an effect of TMR during REM sleep can strengthen memory when using a shock as UCS. If such a design should hint to covert amygdaloid reactivations during REM sleep, a possible, but very challenging, next step would be to further examine the neural correlates implicated in such a reactivation by fMRI. A caveat with this idea is the fact that REM sleep occurs most pronounced during late sleep, in the second half of nocturnal sleep. Problematically, experience showed that participants can only sleep for a maximum of two to three hours in an fMRI scanner. Nevertheless, a solution to conduct such a study could consist for example in selective REM sleep deprivation in participants, which leads to a REM sleep rebound during the next night and thus to earlier REM sleep onset (Beersma, Dijk, Blok, & Everhardus, 1990). This could enable TMR during REM in an fMRI scanner. Importantly, the morning after selective REM sleep deprivation could also be used to study effects of REM sleep absence on emotional processing. However, in such a paradigm retrieval of the previously learned fear would optimally take place only after TMR in the fMRI scanner, since retrieval usually consists in extinction of the CS+.

To sum up, we could not confirm covert reactivations of emotional memory during REM sleep as underlying mechanism behind sleep-dependent emotional re-processing. This could be due to the nature of our task, and future studies are needed to further investigate the role of sleep, and of different sleep stages in emotional memory consolidation and processing.

These studies should be carefully designed and use strong emotional stimuli, such as electrical shocks, to successfully induce emotional learning, and to avoid rather declarative learning.

5.3 Application of the sleep-dependent memory consolidation effect on extinction memory consolidation

In line with our hypotheses, we could show with the fourth study that olfactory TMR of therapy success after *in vivo* exposure-based group therapy in spider phobic patients, compared with odorless vehicle presentation, resulted in an increase in spindle power in response to the reminder cues associated with therapy success. However, unexpectedly, targeting the consolidation process during sleep did not result in a benefit in extinction memory as measured by fear response.

As above described and already replicated with study one, the active system consolidation hypothesis assumes an interaction between slow oscillations, sharp wave-ripples, and spindles in the process of sleep-dependent memory consolidation. Thus, not surprisingly, an effect of TMR was also visible in enhanced spindle power in the fourth study. The active system consolidation hypothesis assumes the above described mechanisms especially during the consolidation of declarative memory. Exposure therapy consists of learning processes encompassing both, declarative and non-declarative memory, although the main proportion is declarative memory. Non-declarative elements are contained especially in the habituation to stimuli associated with the feared object, such as pictures, or the object itself. The larger part of the extinction learning process consists of consciously acquiring new memory traces associated with information about the irrationality of the person's fear, the establishing of clinical behavior due to avoiding the phobic object, and possible alternative behaviors towards the phobic object. Even though this information is acquired in a very emotional context, a clear declarative component is given.

The increases in EEG slow and fast spindle power can be interpreted in line with the active system consolidation hypothesis. In the active system consolidation hypothesis, a key role of sleep in the consolidation of memory by a reorganization of hippocampal information to the neocortex is assumed. The transfer is enabled by an interaction between slow oscillations, spindles, and sharp wave-ripples (Mölle & Born, 2011). As shown in the first study, this mechanism is reflected in specific oscillatory changes in the human EEG in response to TMR, namely in increases in delta activity, slow oscillation slopes, and fast

spindle activity. In study four, there was only evidence for increase in spindle power in response to TMR of therapy success. However, this increase in spindle power might reflect higher efficacy of spindles, for example established by a stronger coupling to sharp wave-ripples, and thus a bottom–up bias of the process of a hippocampal-neocortical transfer.

Importantly, the increase in spindle power was only revealed by an interaction with the amount of activity over the different hemispheres. A possible explanation for this lateralization effect is the strong emotional nature of the exposure therapy, where patients have to face objects they are extremely afraid of. Lateralization of EEG activity is a well-observed phenomenon during learning of emotional tasks (Davidson & Fox, 1982; Ekman & Davidson, 1993).

Contrary to the findings of the first study, a change in the delta power spectrum is not identified by TMR reactivations of extinction memory. Therefore, we did not investigate changes in slow oscillation slopes as done in the first study. A possible speculation about this non-finding is that we used a 1.5 hr-afternoon nap in the fourth study, whereas the sleep period in the first study contained a whole night of sleep. Therefore, we focused on deeper N2 sleep stages to re-present the extinction memory-associated odor, which is also considered as NREM sleep, but with different characteristic electrophysiological oscillations. Of course, we continued the re-presentation of the TMR cues if sleep became deeper and subjects entered SWS, but in the end, the analyzed EEG segments contained N2 and SWS, and not SWS alone, as in the first study.

Interestingly, sleep spindles are the hallmark of N2 sleep, even though they also occur in SWS, whereas slow oscillations are only visible shortly before SWS when N2 sleep gets very deep. The absence of an influence of our TMR on the slow oscillation band could possibly be due to only a small amount of slow oscillations in N2 sleep, whereas spindles are the main oscillatory event of N2 sleep. However, spindles are also included as an important parameter in the active system consolidation paradigm, and a hippocampal bottom-up transfer could possibly be reflected in different oscillatory events based on different NREM sleep stages – slow and fast spindle power in N2 sleep, and delta and fast spindle power in SWS. It is important to underline that this is only a vague speculation and needs to be experimentally verified. A way to do this would be to use the same paradigm of study four with the sleep period extended to a whole night, with TMR only during SWS.

Moreover, the active system consolidation hypothesis, as the sequential hypothesis, speculates about a possible role of REM sleep in system consolidation (Mölle & Born, 2011); that is a consolidation at a cortical synaptic level after reorganization of the information from

the hippocampus to the neocortex. Not surprisingly, only few subjects had REM sleep during their afternoon nap. To analyze an interaction of NREM sleep and REM sleep, more subjects would need to undergo REM sleep during their sleep, which could also be investigated when using a whole night-design.

In contrast to the confirmation of our hypothesis concerning oscillatory changes during TMR, we could not find an effect of TMR on fear behavior after sleep. We explain this finding by a ceiling effect of extinction learning, since exposure therapy is considered very effective in the treatment of anxiety disorders, and very successful even after one session (Öst, Brandberg, & Alm, 1997; Öst, Ferebee, & Furmark, 1997; Öst, 1996). Further, it is important to underline that our patients participated on a self-initiated, voluntary basis and were thus highly motivated and highly committed. Therefore, future studies using less effective paradigms are suggested in order to investigate if TMR can influence fear behavior, such as *in sensu* exposure by watching videos with spiders or only psychoeducation without exposure. Furthermore, since our results indicate also a maximal treatment outcome in the wake group, therapies for other specific phobias that are often treatment-resistant, for example social anxiety disorder (Blanco et al., 2003; Cuthbert, 2002; Heimberg, 2002), could serve as extinction memory learning. In fact, a main reason for the decision to test spider phobic patients in this first clinical application of TMR was the easy access to this patient group because of the high prevalence, usually in addition to little comorbidity. Since our finding constitutes the first attempt to apply basic research to a clinical setting, it would of course be interesting and necessary to replicate the study in a clinical population with another fear disorder.

A possible speculation about memory consolidation during sleep is that post-learning sleep only initiates a process, and thus effects on behavior are not visible immediately, but only after a time. In case of system consolidation, an effective consolidation of the information by a transfer from the hippocampus to existing neocortical networks could probably only be initiated immediately after therapy, and will be more pronounced some time later. In line with this assumption, a previous study found reduced fear after exposure therapy in post-treatment sleep compared with wake patients not immediately after sleep, but one week later (Kleim et al., 2013). To investigate an increase in extinction memory after several time, we designed our study with two sessions separated by one week, and we compared the fear values between sleep groups after this week. But also then, no differences in behavior were found between sleep with reactivation, sleep without reactivation, and wake groups,

which could also be explained by a ceiling effect. Also three months later, subjects of the different groups did not show differences in questionnaires assessing fear of spiders.

Alternatively, the missing effect of TMR could be explained by an ineffectiveness of the reactivation to induce behavioral changes when delta power was not affected by TMR. This could possibly be revealed by the absence of changes in slow wave activity after TMR, reflecting an "incomplete" attempt of memory transfer which could not be finished because of missing time-locked slow oscillations. As a consequence, triggering previously learned declarative information that is still hippocampus-dependent is possible by TMR and entrains spindles, but does not result in preferential consolidation of this information because of the asynchrony of the third key player in this mechanism, slow oscillations.

Since the specific investigation of oscillatory changes was neglected in most human TMR studies, no other studies hint to a causal role of changes in EEG delta power and behavioral changes. This assumption of course needs to be verified by future studies. Also for this research question, a full night of post-extinction learning sleep with TMR could provide answers.

In case future whole night-designs or studies in other fear disorders will uncover an effect of olfactory TMR on extinction memory, it would also be interesting to examine if sounds are also potent as extinction memory cues. This would be important with regard to an application in an everyday clinical setting, since presenting sounds during sleep does not require much equipment, whereas presenting olfactory cues during sleep depends on an olfactometer.

Moreover, given the case that refined paradigms would succeed to find behavioral effects of extinction memory consolidation together with a replication of our EEG findings, a lot of follow-up experiments would be interesting. For example, since an increase in spindles after memory cue presentation seems to reflect the memory consolidation mechanism, spindles during post-learning sleep could also be induced by sinus tones with spindle frequencies, which could lead to an entrainment of the brain in the spindle frequency.

To sum up, the changes in spindle power in response to memory cues seem to reflect targeted extinction memory reactivation on an electrophysiological level. Alternatively, they also could reflect sleep stage-specific effects of TMR during N2 sleep, hallmarked by spindles. Further studies using sleep over a whole night instead of 1.5 hours are needed to investigate these speculations. Moreover, a whole-night paradigm would also be able to account for the role of REM sleep in the active system level consolidation during sleep, a question we did not investigate in study one. Furthermore, our paradigm could be refined by

using patients with different, more treatment-resistant anxiety disorders or less intense treatments for anxiety disorders to prevent ceiling effects, which opens the possibility to enhance the extinction memory.

5.4 Affective disorders and sleep deprivation

While we investigated the mechanisms of sleep-dependent emotional processing and emotional memory consolidation in the third study, we examined why the absence of sleep is an effective treatment in depression, a disorder marked by dysfunctional emotional processing. In line with our hypothesis, we found a reduction in the connection between posterior cingulate cortex and anterior cingulate cortex (ACC) after sleep deprivation compared with normal sleep. In contrast, contrary to our hypothesis, we found increased connectivity between the dorsal nexus (DN) and the dorsolateral prefrontal cortex (DLPFC) after sleep deprivation.

These results demonstrate why sleep deprivation is effective in depression. First, the ACC was found to be a key structure in depression, as shown by hyperconnectivity of the ACC to other brain regions in depressed patients (Greicius, Supekar, Menon, & Dougherty, 2009), which was also associated with rumination (Zhu et al., 2012). Reduction of connectivity to the ACC could thus reduce depressive symptoms. Second, besides this the DLPFC was also shown to be involved in depression, as evidenced by a correlation of a relief of depression symptoms and altered DLPFC activity (Benedetti et al., 2007). We did not expect an increase in connectivity between the DN and the DLPFC. However, since the DLPFC is also strongly involved cognitive control (Miller & Cohen, 2001) this result together with ACC hyperconnectivity could be interpreted as a shift in sleep-deprived resting state activity from brain regions associated with dysregulated affect to more cognitive brain regions.

It should be underlined that this study served as a first investigation to determine effects of the role of sleep and sleep deprivation in emotional processing in depression. We conducted this study with healthy participants because scanning situations and experiment participation are known to cause enormous stress in patients. Moreover, patients are difficult to recruit and show high drop-out rates in studies. Therefore, this pilot study was conducted in healthy participants to examine if sleep deprivation is associated with altered resting state brain activity at all. Since we found very promising results that can account for the effectiveness of sleep deprivation in depression, the study should be applied to depressive

patients in a second step. We expect a replication of the above mentioned results, since the evolved neural correlates could account for the reduction of depressive symptoms. Hereby, it could be of importance to carefully assess the depressive symptoms by regular reports before and after sleep deprivation. It would be interesting to examine whether the strength of changes in resting state connectivity predicts the extent of relief in symptoms. Moreover, an assessment of EEG activity during nocturnal sleep before sleep deprivation could reveal if the change in certain candidate sleep parameters, such as total sleep time, sleep latency, awakenings after sleep onset, REM sleep latency, and REM density are associated with the reduction in depression symptoms after sleep deprivation and the strength of the reduction in ACC connectivity or the increase in DLPFC connectivity, respectively. With these analyses, the question could be further investigated if the effectiveness of sleep deprivation can be explained by its disruption of dysfunctional sleep. In this case, changes in brain activity and depressive symptoms after sleep deprivation should be correlated with the degree of sleep dysregulation. As a next step, it would also be interesting to compare resting state activity during sleep between normal subjects and depressive participants. However, it seems an extremely challenging, if not infeasible, study idea to measure sleep in depressed patients in an fMRI scanner.

5.5 Conclusion and future directions

Altogether, the findings of the five manuscripts give new insights in the mechanisms of sleep-dependent declarative memory consolidation and confirm the active system consolidation hypothesis as underlying neural mechanism involved in this process. They further hint to the same processes of sleep-dependent re-processing in extinction memory consolidation. However, we could not confirm a similar mechanism including covert amygdala-reactivations during REM sleep for emotional memory consolidation and emotional processing. Moreover, we could also detect changes in resting brain activity after sleep deprivation in healthy participants that could explain the effectiveness of sleep deprivation as a treatment in major depressive disorder. As already described in detail in the previous parts of the discussion, our results stimulate a lot of other possible studies. These future directions will be summed up in the following sections.

Future studies based on the first manuscript would need to replicate our conclusion that the memory-enhancing interaction of slow oscillations, sharp wave-ripples, and spindles can be triggered in a bottom-up way. Optimally, single cell recordings in animal studies could

provide insight here. Concerning the effectiveness of TMR, study two would need to be replicated with a memory-associated TMR stimulus. Moreover, a comparison between the behavioral effectiveness and the involved neural correlates of memory-associated olfactory and auditory TMR cues would be interesting.

Especially for the role of sleep in emotional memory processing, future studies are needed. These studies should modify our design by using more intense emotional stimuli, such as electrical shocks. Moreover, they should investigate not only REM sleep, but also SWS as possible key player in emotional memory consolidation. This is certainly a challenge, since emotional memory consists of declarative and non-declarative parts that are not easy to disentangle. Therefore, these tasks need to be designed carefully. In case of results confirming emotional memory consolidation by TMR during REM sleep, in a next step, it would be interesting to identify the neural correlates involved in this replay by fMRI.

Future research is also needed to verify the promising results of study four, as we were the first to examine TMR of extinction memory in patients. Replications especially need to use other paradigms not resulting in a ceiling effect of extinction learning, to examine TMR effects on behavior. This could be achieved by anxiety patients with a high treatment-resistance, or by *in sensu* treatments. Furthermore, studies including a whole night of sleep and TMR only during SWS could provide an insight into the question if this would result in the same findings as in study one. Moreover, the role of REM sleep in system memory consolidation could be investigated with a whole night-design.

Since the fifth study is only a pilot study in healthy participants, a replication of this study in depressive patients would be needed to examine if resting state brain activity changes associated with the ACC and the DLPFC after sleep deprivation are also observed in depressive patients. Hereby, it would be interesting to assess depressive symptoms and sleep before and after sleep deprivation, to see if the strength in resting state changes correlates with these parameters.

To sum up, the mechanisms underlying the beneficial effect of sleep for declarative memory need to be further investigated in other memory forms by the use of TMR which efficiently biases declarative memory replay. Especially the application of TMR to extinction memory gives first hints of an involvement of similar mechanisms in extinction and declarative memory consolidation. The knowledge about underlying mechanisms of extinction memory consolidation holds great potential for understanding sleep-dependent mechanisms of anxiety disorders, and future results could help optimizing treatment outcomes.

6 References

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09/2005 – 07/2008	**Bachelor of Science in Psychology** Major: Biopsychology University of Luxemburg, Luxemburg
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03/2014 – 11/2014	**Research scholar** at the Cognitive Neuroscience Laboratory and the Jay Gottfried Laboratory, Northwestern University, IL, USA

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SKILLS PROFILE

Profound knowledge in biological psychology (sleep, memory, emotional processing)

Knowledge of scientific methods: Conduction and analysis of studies using fMRI, EEG and psychophysiology, peer mentoring 'Psychophysiology', SPM courses in Hamburg and Zurich

Presentation techniques: Poster presentations at national and international congresses, oral presentations, teaching experience

Writing skills: Successful grant applications, publications in international journals

TEACHING EXPERIENCE

02/2013 – 05/2013	Seminar 'Sleep and Memory' for Master students at the University of Zurich, Switzerland

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PUBLICATIONS

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